THE THERMOTROPIC BEHAVIOR OF AQUEOUS DISPERSIONS OF MIXED-ACID LECITHINS

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The Thermotropic Behavior of Aqueous Dispersions of Mixed-acid Lecithins

by

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ABSTRACT

Most phospholipids found in biological membranes are mixed-acid phospholipids. Yet there is little known about the physical properties of mixed-acid phospholipids in bilayers. In this work positional isomers were synthesized using modifications of published procedures. Differential scanning calorimetry was used to study the thermotropic properties of aqueous dispersions of 1-oleoyl-2-palmitoyl-sn-glycero-3-phosphocholine (OPPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-oleoyl-2-stearoyl-sn-glycero-3-phosphocholine (OSPC), 1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine (SOPC), 1-oleoyl-2-arachidoyl-sn-glycero-3-phosphocholine (OAPC), and 1-arachidoyl-2-oleoyl-sn-glycero-3-phosphocholine (AOPC). The gel to liquid-crystalline phase transition temperatures of these lecithins were different from each other, and from those of the parent single-acid lecithins. For each pair of positional isomers the isomer with the longer chain at the sn-1 position had the lower transition temperature.

The thermotropic behavior of mixtures of cholesterol with four mixed-acid lecithins and three single-acid lecithins was studied using differential scanning calorimetry. Asymmetric endotherms were observed, and these were resolved into a narrow and a broad component. The narrow components disappeared at different levels of cholesterol for the different lecithins. The results suggested that for the OSPC-

SOPC pair, cholesterol interacted more with OSPC than with SOPC. For the OAPC-AOPC pair, the interaction was greater between AOPC and cholesterol than between OAPC and cholesterol. Structural differences in the hydrocarbon region of the lecithin bilayers may account for these observations.

Phase diagrams were constructed for mixtures of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) with some mixed-acid lecithins. The phase diagrams were analyzed using Regular Solution Theory, and excess interaction energies were obtained.

Gel phase immiscibility was observed in the mixtures of DPPC with OPOC and with SOPC, but not in mixtures of DMPC with POPC or with 1-palmitoyl-2-stearoyl-sn-glycero-3-phosphocholine. The miscibility in the gel phase appeared to be related to the transition temperatures of the lipids. In the liquid-crystal there was complete miscibility in all four mixtures. The estimates of excess interaction energies suggested that transient clusters of like molecules in the liquid-crystal may occur. Computer simulations based on excess interaction energies were also presented.

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I would like to thank Drs. E.A. Barnsley and E.K. Ralph for their helpful advice during the course of this work and the faculty and staff of the Department of Biochemistry for providing an ideal environment for this work. I am particularly grateful to Dr. K.M.W. Keough for his unceasing help and encouragement and to Miss Elaine Boone for many long hours above and beyond the call of duty during the preparation of this thesis. Finally, I wish to thank my wife, Janet, for suffering my long absences with patience and for her encouragement throughout the course of this work.

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ABBREVIATIONS

AOPC : 1-arachidoyl-2-oleoyl-sn-glycero-3-phosphocholine

ATPase : Mg^{++} adenosine triphosphatase

DAPC : 1,2-diarachidoyl-sn-glycero-3-phosphocholine

DMPC : 1,2-dimyristoyl-sn-glycero-3-phosphocholine

DMPE : 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine

DMSO : dimethylsulfoxide

DOPC : 1,2-dioleoyl-sn-glycero-3-phosphocholine

DPH : diphenylhexatriene

DPPC : 1,2-dipalmitoyl-sn-3-phosphocholine

dsc : differential scanning calorimetry(ic)

DSPC : 1,2-distearoyl-sn-glycero-3-phosphocholine

glc : gas liquid chromatography

LUV(s) : Large unilamellar vesicle(s)

LMV(s) : large multilamellar vesicle(s)

K : degrees Kelvin

nmr : nuclear magnetic resonance spectroscopy

OAPC : 1-oleoyl-2-arachidoyl-sn-glycero-3-phosphocholine

OPPC : 1-oleoyl-2-palmitoyl-sn-glycero-3-phosphocholine

OSPC : 1-oleoyl-2-stearoyl-sn-glycero-3-phosphocholine

PA : phosphatidic acid

PC : phosphatidylcholine

PE : phosphatidylethanolamine

PG : phosphatidylglycerol;

PG-Na : sodium salt of phosphatidylglycerol

POPC : 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine

rpm : revolutions per minute

SOPC : 1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine

SUV(s) : small unilamellar vesicle(s)

T_c : transition temperature

tlc : thin layer chromatography

T_{max} : temperature of maximum excess heat capacity

X_{chol} : mole fraction of cholesterol.

I INTRODUCTION

I-1. Nomenclature and Terminology

Throughout this dissertation there are various terms which may be obscure, and it will be of some value to describe them in some detail. Lecithins are referred to as "single-acid" and "mixed-acid" to denote molecules having two identical acyl chains and two different acyl chains respectively. The types of acyl chains, either saturated or unsaturated, are denoted as follows: saturated single-acid lecithins, those having identical saturated chains; unsaturated single-acid lecithins, those having identical unsaturated chains; saturated mixed-acid lecithins, those containing two different saturated chains; and saturated-unsaturated mixed-acid lecithins, those containing one saturated chain and one unsaturated chain. The term "parent single-acid lecithin" is used to denote single-acid lecithin precursors of mixed-acid lecithins (for example DOPC is the parent single-acid lecithin of OSPC, OPPC and OAPC). Dispersions of one lecithin and of a binary mixture of lecithin are referred to as "single-lipid" and "mixed-lipid" systems. These terms do not indicate the nature of the fatty acids of the lipids.

The positions of the fatty acyl chains and the polar head groups on the glycerol "backbone" of the phospholipids are designated using stereospecific nomenclature (IUPAC)

In naming the mixed-acid lecithins, the distribution of the two fatty acids is indicated as follows: 1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine is referred to as stearoyl-oleoyl lecithin or SOPC where: O = oleoyl, S = stearoyl, P = palmitoyl and A = arachidoyl. Single-acid lecithins are abbreviated as follows: 1,2-diarachidoyl-sn-glycero-3-phosphocholine is referred to as diarachidoyl lecithin or DAPC.

In the description and discussion of the behavior of lecithin-cholesterol mixtures, reference is made to components of the endotherms. The endotherms are assumed to be composed of two superimposed excess heat capacity curves and these curves are obtained by deconvolution of the endotherms as described in section II-7B. It is these deconvoluted curves that are referred to as the components of the endotherms.

I-2 Structure and Function of Membranes

A. Origin of the Bilayer Structure Concept of Membranes

The concept of the lipid bilayer structure being the basic feature of biological membranes arose from the early work of Gorter and Grendel (1925). They showed that monolayers formed from lipids of erythrocyte membranes occupied an area that was approximately twice the surface area of the erythrocyte. This led to the conclusion that erythrocytes were bounded by a bimolecular lipid leaflet. X-ray diffrac-

tion studies on lipid-water mixtures (lipids extracted from bovine spinal cord) also suggested that lipids adopt bilayer structures (Bear et al., 1941; Palmer and Schmitt, 1941). This concept was enlarged and refined by Danielli and Davson in the 1930s, who suggested that the protein components were on the polar surfaces of the bilayer (Davson and Danielli, 1934) and was extended by Singer and Nicholson (1972) who developed the fluid-mosaic structure that has gained acceptance as a model for most biological membranes.

B. Models of Biological Membranes

Several types of lipid-water systems have been used as models of biological membranes. One of the earlier popular models used to mimic the behavior of biological membranes was a lipid monolayer formed at an air-water or an oil-water interface. Monolayers have been viewed as being one half of a lipid bilayer, but it must be noted that the use of monolayers as models for membranes ignores potential interactions between components in different sides of bilayer membranes. This difficulty in extrapolating from monolayers to membranes notwithstanding, the use of monolayer models has contributed much useful information that is pertinent to biological membranes.

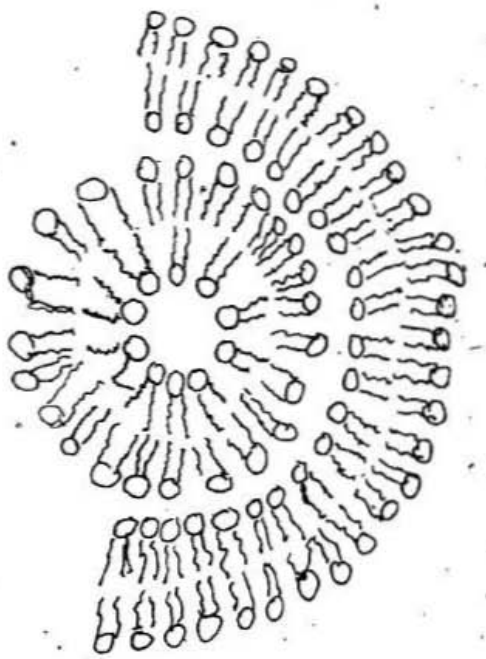
One of the major disadvantages of lipid monolayer models is the fact that monolayers cannot be used to separate aqueous phases, a major function of biological membranes. One model that was used to overcome this

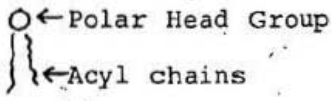
disadvantage was the black lipid membrane formed by depositing a lipid solution in organic solvent at a small pore separating two aqueous compartments and allowing it to thin to a bilayer. These models have been particularly useful in studies of the electrical and permeability properties of lipid membranes' (Mueller et al, 1962; Huang and Thompson, 1966).

By far the most popular models of biological membranes have been the aqueous dispersions of phospholipids developed by Bangham and Horne (1964). This model has been used in this work, and a brief description is included here. Bangham and Horne (1964) found that when phospholipids were shaken in an aqueous medium, they spontaneously form multilamellar structures similar to those illustrated in Figure I-1. These structures are often called "liposomes" or "large multilamellar vesicles" (LMVs). Essentially these structures consist of concentric bimolecular leaflets of phospholipids arranged such that the hydrocarbon chains are excluded from water while the polar head groups are exposed to the aqueous medium. Subsequent work demonstrated that liposome preparations subjected to ultrasonic vibration gave rise to structures consisting of single bimolecular leaflets often called small unilamellar vesicles (SUVs) (Huang et al., 1964). SUVs have also been prepared by removing detergent (sodium cholate) from lipid-detergent mixtures (Brunner et al., 1976). Recently, new techniques have been developed to form large unilamellar (LUVs) by injection of lipid

Figure I-1

The structure of multilamellar vesicles or liposomes (adapted from Bangham and Horne, 1964).




Polar Head Group
Acyl chains

solutions in organic solvent into aqueous media (Deamer and Bangham, 1976).

Multilamellar and unilamellar vesicles have several advantages over other models. They are simple to prepare and, when made with appropriate lipid components, are quite stable. In black lipid membranes the solvent content is uncertain, whereas the lipids used to prepare MLVs and SUVs can be rendered free of solvent before dispersal. Ionic and non-ionic solutes in the dispersing medium will be incorporated into the aqueous medium of both types of vesicles and so permeability parameters can be obtained by changing the external medium after formation of the vesicles and measuring the rate of eflux of the permeants. Various membrane components when mixed with the phospholipid before dispersal are incorporated into the bilayers, and so their effect on the behavior of the bilayers can be monitored.

C. Thermotropic Behavior of Phospholipid Bilayers

Of particular interest, from the point of view of this work, is the thermotropic behavior of hydrated phospholipids in these lamellar structures. There are several important reasons for the widespread interest in the thermotropic properties of phospholipid bilayers that has developed in the last decade or so. Most of the studies on the thermotropic behavior of bilayers have been directed towards understanding the endothermic phase change that occurs in hydrated phospholipid bilayers at temperatures well below the

capillary melting point of the pure lipids. This phase change is often called the "order-disorder" transition or the "gel to liquid-crystalline" transition. The studies of Tardieu et al., (1973) illustrated the basic features of phospholipids in the gel and the liquid-crystalline phases. In the gel phase, the acyl chains exist predominantly in the extended all-trans configuration. There are extensive intermolecular van der Waals interactions and few rotations about the carbon-carbon bonds in the chains. A consequence of this arrangement is a close packing of phospholipids in the bilayer to give a small molecular area in the gel. The closer packing also restricts head group mobility. As the gel is heated above the phase transition temperature the acyl chains become disordered. The number of gauche rotamers increases and chain mobility is enhanced. Consequently the molecular area increases, and the head group also becomes more mobile. The increase in molecular area is coupled with a decrease in the thickness of the acyl chain region (the chains being shortened by the introduction of gauche rotamers) so that the change in molecular volume during the gel to liquid-crystal phase transition is quite small. The ΔV of the gel to liquid-crystal transition of aqueous dispersions of DPPC was about three percent (Nagle, 1973).

One of the most important consequences of this gel to liquid-crystalline phase transition in membranes is a change in the "fluidity" of the membranes. The increased disorder

and chain mobility in the liquid-crystal impart an increased fluidity to membranes. It was shown some time ago that organisms alter the fatty acid composition of their phospholipids in response to changes in growth temperature (Fraenkel and Hopf, 1940). The change in composition with decreasing temperature is consistent with attempts on the part of the organisms to maintain appropriate membrane fluidity (Chapman et al., 1966).

The relationship between the growth temperature and the membrane composition appears to arise from a requirement that membrane lipids possess specific physical characteristics for optimum function of membrane associated enzymes and for the maintenance of normal permeability and transport behavior. For example, Rottem et al. (1973) have shown that a break in the Arrhenius activation energy plots for the membrane ATPase of Mycoplasma mycoides var. capri, adapted to grow without cholesterol, correlated with the gel to liquid-crystalline phase transition temperature detected by differential scanning calorimetry. A discontinuity in the microviscosity measured by fluorescence polarization with diphenylhexatriene (DPH) was also observed at the same temperature at which the break in the activation energy occurred. No phase transition temperature could be detected in the native strain (the native strain contained 22% cholesterol compared with < 3% in the adapted strain). The Arrhenius plot for the native strain did not show a discontinuity.

Papahadjopoulos et al., (1973) observed that the transition temperatures (T_{max}) of multilamellar vescicles and small unilamellar vescicles prepared with DPPC were also the temperatures at which the vesicles showed the greatest permeability to sodium ions and to sucrose. This suggests that the physical state of membrane lipids might be important in determining the permeability properties of the membranes.

In addition to the relationship between the gel to liquid-crystal phase transition and membrane function, studies of phase transition behavior of different lipids can provide information about the structural organization of membranes. Work in this direction has grown rapidly in the last few years and, with more sophisticated techniques, a great deal of information on membrane structures has been obtained. Some of this information is presented in subsequent sections of this dissertation.

I-3 Previous Work

A. Behavior of Lipid Model Systems Containing a Single Lipid

The effects of the molecular structures of phospholipids on the gel to liquid-crystal phase transition have been studied extensively with a variety of model systems. The temperature of the transition, and the enthalpy change associated with it, depend on the type of acyl chains, on the type of head group and on the position of the acyl chains on

the glycerol backbone of the phospholipids (eg., Vaughan and Keough, 1974; Mabrey and Sturtevant, 1976; Keough and Davis, 1979; Silvius et al., 1979; Seelig et al., 1980; Stumpel et al., 1981; Chen and Sturtevant, 1981; Mason et al., 1981a) The bulk of the work has been done using single-acid phospholipids (those having two identical acyl chains) and it was only recently that the behavior of mixed-acid phospholipids (those having two different acyl chains) has been investigated systematically (Keough and Davis, 1979; Stumpel et al., 1981; Chen and Sturtevant, 1981; Mason et al., 1981b). The current information about the effects of lipid structure on thermotropic behavior is summarized below.

i. Effect of Acyl Chain Length in Single-Acid Phospholipids.

The relationship between the length of acyl chains and the thermotropic behavior of phospholipids was first noted in the early application of dsc to the study of the phase transition temperatures of saturated single-acid phosphatidylcholines (PC) and phosphatidylethanolamines (PE) (Chapman et al., 1967; Phillips et al., 1969). Subsequently, a number of workers studied the effect of chain length in saturated single-acid PC on the transition temperature and transition enthalpy using both low and high sensitivity dsc (eg., Hinz and Sturtevant, 1972; Mabrey and Sturtevant, 1976; van Dijck et al., 1976a; Silvius et al., 1979; Mason et al., 1981a). There is an increase in transition tempera-

tures of PC and PE with increasing chain length but the increase per additional methylene unit is smaller when the chains are longer. The effects of chain length on the transition temperatures of various other phospholipids have been studied and there is a remarkable similarity in the effects of chain length on transition temperature among the various phospholipid classes (Phillips et al., 1969; van Dijck et al., 1976a; Findlay and Barton, 1978). This has led to the suggestion that within each head group class, the inter- and intra-molecular interactions between acyl chains are the major factors which determine the thermotropic behavior of these phospholipids, and that these interactions are common to all saturated single-acid phospholipids. It is noteworthy that there is a linear relationship between the transition temperatures and the enthalpies of transition of saturated single-acid PC (Mabrey and Sturtevant, 1976) which would suggest some common structural feature is responsible for determining the transition temperature and transition enthalpies in these lipids. If the acyl chains are fully extended in the bilayer as suggested by other techniques, the van der Waals interactions between chains increase with chain length and it may be these interactions which determine the thermotropic properties of the PC. If nothing else, the relationship between enthalpy and transition temperature supports the idea that the acyl chains are arranged in a similar way in the phospholipids with different length chains, an important point since many of the studies using

other physical techniques have been done with only a few selected phospholipids.

ii. Effect of Head Group

The nature of the head group also influences the phase transition behavior of phospholipids in aqueous dispersions (liposomes). The transition temperatures of phospholipids having identical acyl chains are lower for PC and sodium salts of phosphatidylglycerols (PG-Na) than for PE, phosphatidic acids (PA) and protonated PG. The sodium salts of phosphatidylserines and deprotonated PG have transition temperatures intermediate between PC and PE (van Dijck et al., 1978; Ranier et al., 1979). The effect of the head group on transition behavior may arise from ionic interactions between charged headgroups and from steric effects of bulkier head groups (Vaughan and Keough, 1974). The finding that PE and PC containing two cis-unsaturated chains have almost identical transition temperatures would suggest that steric effects are important. The cis-unsaturated chains apparently lead to large areas per molecule in the gel state of unsaturated PE and thus, the unsaturated PE are packed like unsaturated PC. The effect of the difference in the size of the polar head groups between PC and PE may be abolished by the increased molecular area occupied by the unsaturated acyl chains.

The transition behavior of sphingomyelins (N-acyl-trans-4-sphingosine-1-phosphorylcholines) have also been

studied (Barenholz et al., 1976, Estep et al., 1979, 1980). The transition temperatures of the sphingomyelins also increase with increases in the length of the acyl chains but the phase behavior of dispersions of some sphingomyelins is more complex than that of PC and PE (Estep et al., 1980).

iii. Effect of the Structure of the Backbone Region

The type of linkage of the acyl chains to the glycerol of phospholipids influences the gel to liquid-crystal transition behavior. Phospholipids containing two ether linkages between the acyl chains and the glycerol have been examined and these have slightly higher transition temperatures (two to six degrees) than the corresponding diester analogues (Abramson, 1970; Vaughan and Keough, 1974; Lee and Fitzgerald, 1980; Bittman et al., 1981).

iv. Derivatives of Chains in Single-Acid Phospholipids

Some modifications of the acyl chains have been shown to decrease the transition temperatures of PC with the exception of the 4,4'-difluoro derivative of DMPC, reported by Sturtevant et al. (1979) to have a slightly higher transition temperature than DMPC. Of the chain derivatives studied, the introduction of a cis double bond causes the greatest difference in transition temperature with respect to the saturated phospholipid. The position of a cis double bond in the chains is an important factor in determining T_c (Barton and Gunstone 1975). In studies of a series of

dioctadec-*cis*-enoyl PC, they found that the transition temperature was lowest when the double bond was located at C9 or C10. Double bonds inserted near the methyl or carbonyl ends of the acyl chains had transition temperatures that were much closer to that of DSPC. The transition temperatures of the dioctadec-*trans*-enoyl PC were higher than those of corresponding *cis* isomers. This indicated that *trans* double bonds caused less perturbation than *cis* double bonds.

Branched chain fatty acyl PC and PC containing fatty acids with cyclopropane rings have also been studied extensively by Silvius and McElhaney (1979, 1980). These have lower transition temperatures than their saturated analogues and the transition temperatures of the *trans* cyclopropane isomers were higher than those of the respective *cis* isomers.

v. Positional Distribution of Acyl Chains in Mixed-Acid PC

The behavior of lecithins containing two different saturated acyl chains (saturated mixed-acid lecithins) has been investigated (Keough and Davis, 1979; Stumpel *et al.*, 1981; Chen and Sturtevant, 1981; Mason *et al.*, 1981b). It was found that the positional isomers of saturated mixed-acid lecithins have different transition temperatures. With the exception of the positional isomers of mixed-acid PC containing decanoate and stearate chains (Mason *et al.*, 1981b) the T_c of all the other isomeric pairs of saturated

mixed-acid PC followed the same pattern. The isomer with the 1-long-2-short chain distribution had the lower transition temperature in each pair. Because of a difference in the conformation in the carbonyl region of the chains, the sn-1 chain has a greater effective chain penetration than does the chain at the sn-2 position (see below). Thus, the difference in the depth of penetration between the sn-1 and sn-2 chains is greater in the 1-long-2-short isomer than in the 1-short-2-long isomer. It was suggested that chain interdigitation might not occur to any significant extent in the mixed-acid PC bilayers in the gel state and hence the long chain in the sn-1 position would contain more gauche rotamers (Keough and Davis, 1979). This would lead to less efficient packing and decreased bilayer thickness in the 1-long-2-short isomer than in the 1-short-2-long isomer. This could account for the lower transition temperature in the 1-long-2-short isomer. Others feel that as the effective chain difference becomes greater, chain interdigitation may become more likely (Chen and Sturtevant, 1981; Mason et al., 1981b). This change from non-interdigitating to interdigitating chains could account for the annomolous T_c of the C10/C18 isomeric pair (Mason et al., 1981b).

B. nmr Studies of Model Membranes

Techniques other than thermal analysis must be employed to determine the molecular motions and the structural features that give rise to the thermal changes detected by

differential scanning calorimetry. Nuclear magnetic resonance spectroscopy (nmr) has been used extensively for this purpose. Some of the information obtained from nmr studies that pertains to the issues involved in the present work are summarized in the following paragraphs.

i. Acyl Chain Inequivalence

The early studies of Seelig and Seelig (1975) showed that the acyl chain inequivalence observed in PE crystals by Hitchcock et al. (1974) also occurred in bilayers. They interpreted the appearance at temperatures above T_c of different signals for deuteriums located at the 2'-carbon of the two acyl chains of specifically labeled DPPC as indicative of different conformations adopted by the sn-1 and sn-2 chains in the region near the ester bonds. The estimated difference between the effective penetration of the sn-1 and sn-2 chains obtained by Seelig and Seelig (1975) was 1.8 Å or 1.5 carbon-carbon bonds (projected on the bilayer normal). The chain inequivalence has been confirmed by laser Raman spectroscopic studies (Gaber et al. 1978), by the X-ray studies of Pearson and Pascher (1979) and by neutron diffraction studies (Buldt et al. 1978). Both Gaber et al., (1978) and Buldt et al., (1978) estimate a difference in chain penetration of 1.5 carbon-carbon bonds while this difference in DMPE and DPPC crystals was estimated to be 3.5 carbon-carbon bonds (Hitchcock et al., 1974; Pearson and Pascher, 1979). The studies of Seelig and Seelig (1975)

were carried out above the transition temperature - i.e., their results reflect the arrangements existing in the liquid-crystal. The studies carried out by Gaber *et al.*, (1978) demonstrated that the chain inequivalence between the sn-1 and sn-2 chains also exists in the gel phase. Another important finding of Gaber *et al.*, (1978) involved the structure of the acyl chains near the terminal methyl groups. These authors state that in DPPC, the terminal methyl chain of the sn-1 chain was more distorted than the same region of the sn-2 chain. This is consistent with the suggestion that, in bilayers of phospholipids containing acyl chains of the same length or chains that differ only by a certain small amount, chain interdigitation is avoided by some foreshortening (possibly by introduction of gauche rotamers) of the long chain at the sn-1 position with a concommittant increase in disorder in the hydrocarbon region (Keough and Davis, 1979; Mason *et al.*, 1981).

ii. Orientation of the Double Bonds.

The nmr study of Seelig and Waespe-Sarcevic (1978) indicated that the average orientation of the $\Delta 9,10$ *cis* double bond in POPC above its transition temperature is approximately parallel (at an angle of 6-8°) to the bilayer normal. Although the orientation of the double bond in POPC below the transition temperatures has not been determined, it would seem reasonable to assume that a similar orientation (nearly parallel to the bilayer normal) to that in the

liquid-crystal would persist into the gel.

C. Studies of Lipid-Cholesterol Interactions.

Cholesterol occurs in significant concentrations in many biological membranes and its interaction with other membrane components, especially the phospholipids, has been the subject of intensive study for many years. The early investigations of the effects of cholesterol on the behavior of phospholipid bilayers and natural membranes have been reviewed recently (see Demel and de Kruijff, 1976). These early studies indicated that cholesterol exerted some dramatic effects on the physical behavior of phospholipids in bilayers. Ladbrooke et al. (1968) reported that addition of cholesterol to simple model membranes composed of synthetic phosphatidylcholines caused a reduction in the magnitude of the gel to liquid-crystalline phase transition. They suggested that cholesterol caused the acyl chains of gel phase phospholipids to beoome more fluid and the chains in the liquid-crystalline phase to become more rigid. They proposed that a function of cholesterol in membranes might be that of a fluidity modulator. It was shown later that the presence of cholesterol in membranes from Mycoplasma mycoides var. capri abolished the gel to liquid-crystalline transition that was observed in membranes from an adapted strain of this organism having almost no cholesterol (Rottem et al., 1973).

Many of the early studies of cholesterol-phospholipid

mixtures were interpreted in terms of cholesterol-lipid complexes and a variety of stoichiometries have been assigned to these complexes. Measurements of the rotation rates of egg PC and cholesterol on their respective axis in sonicated PC-cholesterol vesicles have shown that in the liquid-crystal the rotation of the phospholipid molecules is one or two orders of magnitude slower than that of cholesterol molecules (Yeagle, 1981). This finding was not consistent with the formation of strong complexes between phospholipids and cholesterols in the liquid-crystal. It has also been suggested that many of the stoichiometries assigned to the putative "complexes" may have arisen from metastable lipid-cholesterol phases caused by the method of preparation of the aqueous dispersions (Collins and Phillips, 1982). These authors have suggested that the maximum equilibrium solubility of cholesterol in DPPC bilayers is 1.0 $\pm$ 0.1 mole cholesterol per mole of lecithin.

The electron spin resonance studies of Shimshick and McConnell (1973) indicated the existence of a phase boundary at 20 mol% cholesterol in bilayers of cholesterol plus DPPC or DMPC. The occurrence of phase separation in aqueous dispersions of phospholipid-cholesterol mixtures has received much attention in the last few years. The dsc endotherms of the gel to liquid-crystal phase transition of mixtures of cholesterol and DPPC exhibited a high-temperature asymmetry at $X_{chol} \leq 0.22$ (Estep et al., 1978; Mabrey et al., 1978). These asymmetric endotherms have been

resolved into a narrow low-temperature component and a broad component having a slightly higher T_{max} (Estep et al., 1978; Mabrey et al., 1978). The narrow components disappeared at 22-25mol% of cholesterol and the enthalpies associated with the remaining broad components decreased as the cholesterol concentration increased. The broad components persisted up to about 50mol% of cholesterol (Mabrey et al., 1978).

Asymmetric endotherms have also been observed for mixtures of cholesterol with palmitoylsphingomyelin and lignoceroylsphingomyelin, and these have been resolved into two components (Estep et al., 1979). The endotherms observed by Blume (1980) for mixtures of cholesterol with saturated single-acid phosphatidylethanolamines were asymmetric but, for these mixtures, a low-temperature asymmetry could be observed.

It has been suggested that the narrow component of the asymmetric endotherms obtained for the lecithin-cholesterol and sphingomyelin-cholesterol mixtures most likely arose from phase transitions in a domain of nearly pure lipid (Estep et al, 1978, 1979; Mabrey et al., 1978; Snyder and Freire, 1980). It has also been suggested that the broad components of these endotherms reflect transitions occurring either in a cholesterol-rich domain (Mabrey et al., 1978) or in an interfacial region between a pure lipid domain and a cholesterol-rich domain (Estep et al., 1978, 1979; Snyder and Freire, 1980).

Several models have been proposed for the arrangement of lecithin and cholesterol in lecithin-cholesterol bilayers. The earlier model of Engleman and Rothman (1972) predicted a phase boundary at $X_{chol} = 0.33$ but did not account for the two component endotherms discussed above. The phase boundary at 22-25% cholesterol in DPPC (Estep et al., 1978,; Mabrey et al., 1978) was predicted by the model of Martin and Yeagle (1978) which suggested that three arrangements of lecithin and cholesterol were possible. At $X_{chol} \leq 0.22$ each cholesterol molecule was surrounded by its own separate shell of acyl chains (approximately 3.5 lipids/molecule) with excess lecithin being present. For $0.22 \leq X_{chol} \leq 0.33$, cholesterol exists either as a monomer surrounded by a separate shell of acyl chains or as a dimer with a separate shell of acyl chains, the latter growing at the expense of the former as the cholesterol concentration increases. At $0.33 \leq X_{chol} \leq 47$, dimeric cholesterol having a separate shell of acyl chains coexists with dimers having shared acyl chains, the latter growing at the expense of the former until, at $X_{chol} = 0.47$, only shared acyl chains occur. The model would predict that at $X_{chol} > 0.47$ pure cholesterol would separate out as suggested by Collins and Phillips (1982). Slater and Caille (1981) have suggested that cholesterol dimers having individual shells of acyl chains would be unstable relative to the other arrangements and so that at $X_{chol} < 0.22$ pure lipid would coexist with monomeric cholesterol surrounded by individual acyl chains.

At $0.22 \leq X_{chol} \leq 0.47$ cholesterol monomers having individual acyl chains would coexist with cholesterol dimers having shared acyl chains. The model of Slater and Caille (1981) predicts that the dimeric cholesterol with shared acyl chains does not exhibit a phase transition while the lipids surrounding the monomeric cholesterol have a broad low-enthalpy transition. This modified version (Slater and Caille, 1981), of the Martin and Yeagle (1978) model is very similar to the model proposed by Snyder and Freire (1980). They made three assumptions: 1) there were three types of lipid molecules in the lipid-cholesterol bilayer - lipid adjacent to cholesterol molecules (bound lipids), free lipid, and lipid molecules next to bound lipids (boundary lipids); 2) only the free lipid and boundary lipid contribute to the enthalpy of the phase transition; and 3) the enthalpy of the boundary lipid was the maximum enthalpy observed for the broad component of the phase transition endotherms for lipid-cholesterol mixtures. These authors have obtained good agreement with experimental observations for different lipid-cholesterol mixtures using computer simulations based on the above model.

Although most of the studies of lipid-cholesterol interaction have been done with saturated single-acid lecithins and phosphatidylethanolamines, and sphingomyelins, there have been some attempts to study the effect of cholesterol on mixed-acid lecithins containing saturated and unsaturated acyl chains. Most of these have used egg yolk

lecithin, which is itself a complex mixture of lecithins, and thus, the interpretation of the results is more uncertain. Some pure mixed-acid lecithin-cholesterol systems have been studied in monolayers (Joos et al., 1969; Demel et al. 1972; Ghosh et al., 1973; Stoffel et al., 1974). The monolayer studies of Joos et al., (1969) showed that the interaction energy between PC and cholesterol in monolayers of OSPC-cholesterol was greater than for DOPC-cholesterol indicating a greater interaction in the OSPC-cholesterol mixtures than in the DOPC-cholesterol mixtures.

It was proposed by Huang (1977) that the positional distribution of the fatty acids in saturated-unsaturated mixed-acid lecithins might influence the interaction of cholesterol with these lecithins. Studies by Demel et al., (1972) of the effect of cholesterol on the permeability of liposomes composed of OSPC and those having SOPC did not show any difference between the two positional isomers. These authors have also reported that there were no differences in the molecular areas (at a surface pressure of 12milliNewtons meter^{-1}) between these positional isomers in monolayers of either the pure lecithins or equimolar lecithin-cholesterol mixtures. These studies were carried out above the gel to liquid-crystalline phase transition temperatures and do not give information about the interaction of cholesterol in the gel.

D. Mixing in Binary Mixtures of Lecithins

Biological membranes are complex mixtures of different types of phospholipids, proteins and other components. The distribution of phospholipid classes and the fatty acid composition of the phospholipids from the same organ in different species have been found to be similar. Also the composition of the membranes of specific organelles in different species and in the different organs of a given species show many similarities. Moreover a change in membrane lipid composition is involved in the "homeoviscous" response to changes in environmental conditions. Thus, the complex composition of biological membranes appears to be essential, and it seems likely that the distribution of the lipids in membranes and the interactions between different lipids may play an important role in determining membrane function. Thus a thorough understanding of the structure and function of biological membranes will require information about the nature of the interactions between different membrane phospholipids and the distribution of these phospholipids. One approach to this problem that has been widely used is the study of model binary mixtures of synthetic phospholipids in aqueous dispersions. Silvius (1982) has summarized most of the results for binary mixtures studied to date. A few of the binary mixtures of different lecithins and some binary mixtures of lecithins with phosphatidylethanolamines have been subjected to quantitative analysis and estimates of the differences between the

interaction energies of like molecules and those of unlike molecules (excess interaction energies) have been obtained (Lee, 1977, 1978; von Dreele, 1978; Freire and Snyder, 1980; Cheng, 1980). The approaches of Lee (1977, 1978) and Cheng (1980) are based on "regular solution theory" (Hildebrand and Scott, 1964) while the analyses of von Dreele (1978) and Freire and Snyder (1980) use a statistical approach. It should be noted that these two approaches differ in the assumed origin of the excess interaction energies. Regular solution theory assumes that the excess interaction energy arises from the difference between the enthalpies of mixing of like molecules and unlike molecules (i.e. $\Delta S^{EXCESS} = 0$). In the statistical analyses of von Dreele (1978) and Freire and Snyder (1980) the excess interaction parameter is obtained from estimates of the randomness of the distribution of the different molecules (i.e., $\Delta S^{EXCESS} \neq 0$). In spite of the difference in the assumptions required for these two approaches, the estimates of the "non-ideality" of mixing of the binary mixtures that were analyzed by the two approaches are similar.

The general finding of the analyses of the behavior of binary mixtures of phospholipids is that the mixing of phospholipids decreases in both the gel and liquid-crystalline phases as the differences in the transition temperatures and the acyl chain lengths between the two components increases. It has been suggested before that lateral phase separation may occur in biological membranes (Oldfield *et al.*, 1972)

and the studies of binary lipid mixtures suggest that differences in the molecular interactions could be responsible for such phase separation.

It is notable that these have been studies of mixtures of single-acid phospholipids. Biological membranes contain many different types of phospholipids, and the majority of these contain two different acyl chains (mixed-acid lipids). Part of this work attempts to obtain information about the mixing behavior of such mixed-acid phospholipids in simple binary systems and about the influence of the structure of the acyl chains on this behavior.

I-4 This Work

A. Objectives

The major objectives of this work were to investigate the thermotropic behavior of mixed-acid lecithins containing a saturated acyl chain and an unsaturated acyl chain and the mixing behavior of these mixed-acid lecithins with cholesterol and with some saturated single-acid lecithins. Of particular interest was the effects of small structural modifications in the acyl chains on the behavior of the mixed-acid lecithins and the consequences of changing the positional distribution of the two acyl chains in each mixed-acid lecithin.

B. The Approach

As mentioned before, mixed-acid lecithins containing saturated and unsaturated chains were not available from commercial sources and, thus, part of this work involved studies of various synthetic procedures for the preparation of these lecithins to determine the procedures that, in this laboratory, gave the best products with good yields. The extent of formation of reversed positional isomers during the synthesis was the major criteria by which each of the procedures were evaluated.

The study of the behavior of the pure lecithins and of the mixtures of the lecithins with either cholesterol or with other lecithins were done using differential scanning calorimetry. Two of the major advantages of this technique are the absence of probe molecules which might perturb the system being studied and the fact that dsc provides the enthalpies of the gel to liquid-crystal phase transitions which are required for the analyses of both the lecithin-cholesterol mixing and the phase diagrams for the binary mixtures of lecithins. In this way, differential scanning calorimetry is a particularly useful technique for the studies involved in this work.

The analytical procedures that are applied to the data obtained with lecithin-lecithin and lecithin-cholesterol mixtures have been reported by others and the application of these procedures, their relevance to this work, and their

limitations are discussed in subsequent sections of this dissertation.

II MATERIALS AND METHODS

II-1 Reagents and Solvents

Lecithins were synthesized as described below or were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A., or from Calbiochem-Behring Corporation, La Jolla, CA, U.S.A. Cadmium chloride complex of glycerophosphoryl choline, carbodiimidazole, cholesterol, dicyclohexylcardodiimide, dimethylaminopyridine, dimethylsulfoxide and fatty acids were obtained from Sigma Chemical Company. Cadmium chloride was purchased from Aldrich Chemical Company (Canada) Ltd., Montreal, PQ. Silica Gel G-60 was a product of E. Merck, Darmstadt, Germany. Silicic acid (100-120 mesh) was made by Fluka AG, Buchs, Switzerland. Whatman CM-52 carboxymethyl cellulose was purchased from Mandel Scientific, Rockwood, ON, Canada. Other reagents (ACS grade) were obtained from Fisher Scientific, Halifax, NS, Canada. Chloroform (ACS) and methanol (ACS) were obtained from Fisher and were glass distilled before use. Chloroform (99mol% pure) and methanol (99.9mol% pure) were also obtained from Fisher. Ethanol (95% and absolute) were purchased from Consolidated Alcohols Ltd., Toronto, ON, Canada.

Fatty acids were analyzed by gas liquid chromatography, found to be greater than 99% pure and were used without further purification. Lecithins were analyzed by thin layer chromatography as described below and showed a major spot that corresponded to 1,2-diacyl-sn-glycero-3-phosphocholine.

Some preparations also showed a minor spot that corresponded to 1,3-diacyl-sn-glycero-2-phosphocholine. This minor spot contained less than one percent of the total lipid phosphorus applied to the chromatographic plates.

Cholesterol was recrystallized twice from 95% ethanol at 4°C. It was dried in vacuo over phosphorus pentoxide for at least 16 hours and was stored at -20°C.

II-2 Synthesis of Single-Acid Lecithins

A. Preparation of L-α-glycerophosphorylcholine (GPC)

Glycerophosphorylcholine was prepared essentially as described by Brockerhoff and Yurkowski (1965) from egg yolk lecithin (EYL). EYL was dissolved in diethyl ether (0.1g/ml) and was treated with 25% (w/v) tetrabutylammonium hydroxide in methanol (1 ml/g EYL) for one hour at room temperature (20-25°C). The GPC appeared as a viscous brown-white oil on the sides of the flask. The solvent was decanted and the residue was washed three times with diethyl ether. The GPC was evacuated over phosphorus pentoxide for at least 16 hours before use.

B. Preparation of Fatty Acid Anhydrides

Fatty acid anhydrides were prepared according to the procedure of Selinger and Lapidot (1966). Two millimoles of fatty acid in 15 ml of dry carbon tetrachloride were added to one millimole of dicyclohexylcarbodiimide in 5 ml of car-

bon tetrachloride. The mixture was shaken and left at room temperature for five hours. The insoluble dicyclohexylurea was removed by vacuum filtration (Whatman #1) and the filtrate was evaporated to dryness on a rotary evaporator at 40-50°C. The product was recrystallized from dry acetone (6.25 ml per millimole of fatty acid) at 0°C to remove dicyclohexylacylurea. The crystals were collected by filtration (Whatman #1) and were dried *in vacuo* over phosphorus pentoxide until used. Anhydrides were prepared fresh for each synthesis.

C. Acylation of GPC

i. Two different acylation procedures were used to prepare single-acid lecithins. The first of these was a modification of the procedure of Cubero-Robles and van den Berg (1969) as summarized by Keough and Davis (1979). GPC (1 mmol) was dried onto the walls of a round bottom flask of sufficient size to provide a large surface area (eg. one mmol GPC in a 500 ml flask). Six mmol of fatty acid anhydride were added and the flask was rotated in a heated oil bath to melt the anhydride (65-70°C for oleoyl and palmitoyl anhydride, 85-95°C for stearoyl anhydride). One-half mmol of sodium oxide (98% pure from Alfa Products, Beverly, MA, U.S.A.) was added to the molten mixture and the flask was rotated in the oil bath for 16-24 hours. The mixture was cooled to room temperature and was dissolved in the minimum volume of boiling chloroform. The solution was cooled to

-20°C and insoluble material was removed by vacuum filtration (Whatman #1) at room temperature. The volume of the filtrate was reduced to a minimum and the crude lecithin was precipitated by addition of four volumes of acetone at -20°C. The precipitate was collected by centrifugation as described below, and was taken up in a small volume of chloroform.

ii. The second acylation procedure used was a small modification of the procedure of Gupta et al. (1977). GPC·$CdCl_2$ was dissolved in anhydrous chloroform. The anhydrous chloroform was prepared by shaking redistilled chloroform several times with fresh phosphorus pentoxide until the phosphorus pentoxide no longer formed clumps. The chloroform was distilled from fresh phosphorus pentoxide onto 4A molecular sieves activated at 110°C for several hours and cooled before use. One millimole of GPC·$CdCl_2$ was dissolved in 25 ml of anhydrous chloroform. Fatty acid anhydride (2.3 mmol) and dimethylaminopyridine (2.0 mmol) were added. The flask was flushed with N_2 gas and sealed tightly (ground glass joint). The mixture was stirred at room temperature for 30-36 hours. In some preparations a yellow-green color appeared at the start of the acylation. This color faded with time and its appearance did not affect the quality of the preparation. The color was removed in subsequent purification. When the acylation reaction was completed, the solvent was removed on a rotary evaporator at 35°C and the residue was taken up in 20 ml of chloroform:methanol:water,

4:5:1 (v/v/v). Insoluble material was removed by filtration (Whatman #1) and the filtrate was applied to a Rexyn I-300 (Fisher Scientific) mixed-bed ion exchange column. Rexyn columns were prepared by suspending Rexyn beads in chloroform:methanol:water, 4:5:1 (v/v/v). The suspension was poured into a glass column fitted with a glass wool plug. The column was washed with three to five bed volumes of the same solvent. Entrapped gas bubbles which formed during this process were removed by gently tapping the column. The column was covered with aluminum foil to exclude light and was used immediately after preparation. Columns left in this solvent for more than a few hours changed color and were no longer effective. Column bed volume was 50 ml per mmol of GPC used. The crude lecithin was eluted with two to three bed volumes of the same solvent. The composition of the eluant was adjusted to chloroform:methanol:water, 2:1:0.6 (v/v/v) (Folch et al., 1957). The lower phase which separated was collected, and the solvent was removed on a rotary evaporator at 45°C. The last trace of water was eliminated by successive evaporations with small volumes of dry benzene or diethyl ether and the residue was evacuated over phosphorus pentoxide for at least two hours.

II-3 Synthesis of Mixed-Acid Lecithins

The synthesis of mixed-acid lecithins involved the removal of the fatty acyl chain esterified at the sn-2 posi-

tion of a single acid lecithin. The 1-acyl-lysolecithin was reacylated using fatty acid anhydride or fatty acid imidazolide.

A. Preparation of Lysolecithin

1. Two different procedures were used for the preparation of lysolecithin. One of these was developed in this laboratory (Keough and Davis, 1979). One gram of single-acid lecithin was dissolved in methanol (7.5 ml), chloroform (3.8 ml) and diethyl ether (64 ml). Two-ml of phospholiphase A_2 solution (12 mg Crotalus adamanteus venom, Miami Serpentarium, Miami, FL, U.S.A., in one ml of 10mM $CaCl_2$) was added. The mixture was shaken vigorously and additional two-ml aliquots of enzyme solution were added at 10, 30 and 60 minutes. At 75 minutes a sample of the reaction mixture containing approximately 40µg lipid phosphorus was applied to a tlc plate and the plate was developed for phospholipids (see below). Shaking of the mixture was continued while the plate was developed. If a lecithin spot was present the mixture was shaken until subsequent tlc checks did not indicate the presence of lecithin. If no lecithin spot could be detected after staining the tlc plate with I_2 vapor, the reaction was stopped by adding 50 ml of ethanol or methanol. The solvents were removed on a rotary evaporator at 45°C and residual water was eliminated by several evaporations with diethyl ether. The lysolecithin was washed several times with diethyl ether and was evacuated over phosphorus

pentoxide for 4-16 hours. To confirm the absence of any single-acid lecithin in the lysolecithin preparation, silica gel was scraped from the lysolecithin spot, from the plate origin and from the area corresponding to the position of a lecithin spot and the amount of lipid phosphorus in each of the three gel samples was determined as described below.

ii. A second procedure for the preparation of lysolecithin was essentially as described by Chakrabarti and Khorana (1975). Single-acid lecithin (1.2 mmol) was dissolved in methanol (12 ml) and diethyl ether (196 ml). Enzyme solution (5 mg Crotalus adamanteus venom in 49 ml of 100mM borate buffer, pH7.4, containing 0.72mM $CaCl_2$) was added. The mixture was stirred vigorously at room temperature for 90 minutes to 3 hours until no lecithin spot was detected on tlc plates stained with I_2 vapor. The aqueous layer was collected, washed twice with diethyl ether, and lyophilized. This process required 16 to 30 hours, depending on the volume of frozen suspension. The dry material was extracted twice with 100 ml of chloroform:methanol (2:1 by volume). The pooled extracts were evaporated to dryness on a rotary evaporator at 45°C and the residue was evacuated over phosphorus pentoxide for 4 to 16 hours.

B. Acylation of Lysolecithin

i. Three different acylation procedures were used in the course of this work. The first of these was the modification of the procedure of Cubero-Robles and van den Berg

(1969) as described above (Section II-2Ci; Keough and Davis, 1979). In the acylation of lysolecithin the lysolecithin-anhydride- Na_2O mixture was rotated in the heated oil bath for six hours.

ii. In the second acylation procedure, lysolecithin or its cadmium chloride adduct (lysoPC·$CdCl_2$) was dissolved in anhydrous chloroform. The acylation was according to the procedure of Gupta et al., (1977) modified as described in Section II-2Cii. The molar ratio of reactants was lysolecithin:anhydride:dimethylamminopyridine, 1:2.3:1.

iii. The third acylation procedure was essentially that of Warner and Benson (1977) for synthesis of single-acid lecithins. Dimethylsulfoxide (DMSO) was distilled from calcium hydride onto activated molecular sieves before use. One millimole of lysolecithin was dissolved in 24 ml of dry DMSO with gentle warming (50-60°C). The solution was cooled to room temperature and four millimoles of fatty acid imidazolide in four-ml of DMSO were added. Imidazolides were prepared by shaking fatty acid (4.0 mmol) and carbodiimidazole (4.4 mmol) in DMSO (4.0 ml) for 45 minutes. A slight green color developed on addition of the fatty acid imidazolide. Sodium methylsulfinylmethide was prepared by reacting sodium metal (8.8 mmols) with DMSO (14.4 ml) under N_2 gas until no sodium remained. This was added to the lysolecithin-imidazolide mixture and stirred at room temperature for 5 minutes. The mixture was cooled in ice and

the pH was adjusted to pH4-5 with HCl. The lecithin was extracted with three aliquots of 50 ml of choloroform:methanol, 2:1 (by volume) and the pooled extracts were backwashed twice with 20 ml of methanol:water, 1:1 (by volume). The extract was reduced to dryness on a rotary evaporator at 45°C and the residue was taken up in chloroform.

II-4 Purification of Synthetic Lecithins

A. Silicic Acid Column Chromatography

A slurry was prepared by suspending a 1:1 (by weight) mixture of silicic acid (100-120 mesh) and Hyflo SuperCel (Fisher) in excess chloroform. The slurry was poured into a glass column fitted with a glass wool plug and a ground glass or teflon stopcock. The excess solvent was allowed to drain off and the crude lecithin in chloroform solution was applied to the column. The sample was washed onto the column with small volumes of chloroform. The column was eluted with the following solvents: chloroform - 5 bed volumes (collected as one fraction); chloroform:methanol, 9:1 (by volume) - 5 bed volumes (collected as one fraction); chloroform:methanol, 8:2 (by volume) - 10 to 15 bed volumes (collected as one half bed volume per fraction), until no lecithin could be detected in the column eluant. Fractions containing organic phosphorus were checked by tlc. Fractions containing only lecithin were pooled. The solvents were removed on a rotary evaporator at 45°C and the residue

was taken up in chloroform. The chloroform solution was filtered through a double Millipore type-HA filter (pore size 1.2 µm above 0.47µm) to remove silicic acid fines.

B. Carboxymethyl Cellulose (CM-52) Column Chromatography

Purification on CM-52 columns was essentially as described by Cumfurius and Zwaal (1977). Whatman CM-52 carboxymethyl cellulose was washed ten times by stirring in excess methanol, allowing the cellulose to settle and decanting the excess solvent and fines. The cellulose was resuspended in methanol and poured into a glass column fitted with a glass wool plug. A second glass wool plug was placed on top of the column and this was covered with glass beads. The column was washed with ten bed volumes of chloroform. Crude lecithin solution in chloroform was applied to the column (5 mg lipid per ml bed volume) and it was washed on with a small volume of chloroform. The column was eluted as follows: chloroform - 10 bed volumes (collected as one fraction); chloroform:methanol, 99:1 (by volume) - 3 bed volumes (collected as one fraction); chloroform:methanol, 98:2 (by volume) - 5 bed volumes (collected as one fraction); chloroform:methanol, 95:5 (by volume) - 20 bed volumes (collected as one half bed volume fractions); chloroform:methanol, 75:25 (by volume) - 3 bed volumes (collected as one fraction); methanol - 3 bed volumes (collected as one fraction). The columns were reactivated by washing with ten bed volumes of chloroform. The

column material was discarded after being used three or four times.

C. Lobar Chromatographic Columns

The application of Lobar prepacked columns, from E. Merck, Darmstadt, Germany, to the purification of phospholipids was described by Radin (1978). Two column sizes were used (B-size columns were 25 X 310 mm and C-size columns were 37 X 440 mm). The columns were packed with Lichroprep Si-60 silica gel (Merck). The guard columns described by Radin (1978) were not used. The lecithin preparation was dissolved in hexane:isopropanal:water, 60:80:12 (v/v/v) and the solution was injected onto the Lobar column using a glass syringe. The lecithin was washed onto the column with 100 ml and 600 ml of this same solvent for the B-size and C-size columns respectively. Loads were 1.4-1.9g of lecithin on B-size columns and up to 7g on C-size columns. Loading of distearoyl lecithin required slight warming of the loading tube and syringe to prevent precipitation of the lecithin preparation. Columns were eluted with 1.0 to 1.5 liters of hexane:isopropanal:water, 60:80:15 (v/v/v) for B-size columns and with 3 liters of this solvent for C-size columns. Solvent was pumped at a rate of 5-10 ml per minute using a Constametric II solvent delivery system manufactured by Laboratory Data Control, Riviera Beach, FL, U.S.A. Fractions of 20 ml were collected from B columns, and 50 ml fractions from C columns. Fractions containing phosphorus

were checked by tlc. Those having only lecithin were pooled, the pooled extract was reduced to dryness on a rotary evaporator at 50°C and the residue was evacuated over phosphorus pentoxide for 16 hours.

D. Acetone Precipitation of Lecithin

Lecithin was dissolved in a minimum volume of chloroform. Four volumes of acetone were added and the solution was cooled to 0°C for lecithins having saturated acyl chains and to -20°C for lecithins having one monoenoic chain. The white precipitate was collected by centrifugation at 1500 rpm for 10 minutes in an IEC clinical centrifuge. The centrifuge tube holders were precooled to -20°C to prevent the solutions from warming too quickly. The supernatant was removed and the precipitate was redissolved in chloroform. Two or three precipitations were done routinely on all lecithin preparations, except those having two unsaturated acyl chains.

E. Crystallization of Lecithins

Lecithin was dissolved in hexane:methanol, 98.2 (by volume) to make a 2% (w/v) solution. One mole of water (glass distilled from alkaline potassium permanganate) was added for each mole of lipid phosphorus and the solution was stored at -20°C for three weeks to two months. Microcrystals were collected by centrifugation at 5000 rpm for 10 minutes in an IEC 870 rotor cooled to 0°C. The supernatant

was decanted and the pellet was taken up in 99mol% chloroform.

II-5 Analytical Techniques

A. Preparation of Thin Layer Chromatographic Plates

Glass plates (200 X 200 X 4 mm) were washed in phosphate free detergent (Extran-300, Fisher Chemicals) and soaked in chromic acid for several hours. The plates were rinsed well in tap water and in distilled water. Plates were dried at 110°C for 1-2 hours. A slurry was prepared with 35g of Silica Gel G-60 and 80 ml of distilled water by mixing in a Waring Blender (model 5011) at high speed for one to two minutes. The slurry was spread on clean dry plates, that had been wiped with a Kimwipe soaked with methanol, using a Desaga template and spreader adjusted to give a 0.25 mm thick layer of gel. The time for preparation of the slurry and the spreading of the plates was less than five minutes. The plates were covered to prevent dust contamination and were air-dried.

B. Thin Layer Chromatography

Silica Gel G-60 plates prepared as described above were activated at 110°C for 45 to 60 minutes. The plates were allowed to cool to room temperature. Lanes (usually 2 cm wide) were scored in the gel surface. This generally improved the reproducibility of the mobility of components in various spots across the plate. Samples containing 0.5

to 1.0 mg of lipid were applied in thin bands using a micro-syringe. Plates were developed in one of the following solvent systems: a) for phospholipids - chloroform:methanol:water, 65:25:4 (v/v/v), according to Wagner et al. (1961); b) for neutral lipids - hexane:diethyl ether:acetic acid, 90:10:1 (v/v/v) according to Breckenridge and Kuksis (1968); c) for water soluble components - methanol:water, 70:30 (by volume) from Chada (1970).

C. Detection of Chromatographic Spots

Chromatographic spots were detected using one or more of the following: a) spraying with a 0.2% (w/v) solution of dichlorofluorescein in ethanol (viewed under U.V. light): b) exposure to I_2 vapor; c) charring at 180°C overnight after spraying with 70% (v/v) sulfuric acid; d) spraying with distilled water (lipid spots do not wet); e) spraying with modified Dittmer-Lester reagent (Ryu and MacCoss, 1979), which is specific for lipids containing phosphorus; f) spraying with Hanes-Isherwood reagent (Hanes and Isherwood, 1949), which is also specific for lipids containing phosphorus.

D. Gas Liquid Chromatography (glc)

Methyl esters of fatty acids were prepared by heating lipid samples or material scraped from tlc plates with two ml of 6% (by volume) sulfuric acid (reagent grade) in

99.9mol% methanol containing a crystal of hydroquinone per 25 to 100 ml as an antioxidant. Samples were heated at 80-85°C for 16 hours in sealed Pyrex thick-walled ignition tubes. The tubes were cooled to room temperature, and were opened. Distilled water (1-ml) was added and the methyl esters were extracted three times with 1.5-ml of hexanes (Fisher, pesticide grade). The pooled extracts were washed with one-ml of distilled water and were dried in a stream of N_2 gas at 30-35°C. The residue was taken up in carbon disulfide.

Coiled glass columns (182 X 0.2 cm) were obtained from Applied Science Inc., State College, PA, U.S.A. Columns were washed with chloroform, treated with dichlorodimethylsilane (Applied Science Inc.) and dried at 110°C for several hours. Columns were packed with 10% (by weight) EGSS-X (ethylenesuccinate-methylsilicone copolymer) on Gas Chrom P obtained from Applied Science Inc. Silicone rubber septa were fitted and the columns were installed in a Packard 7400 series gas chromatograph.

Samples in carbon disulfide (1-2.5µl) were injected into the glc column inlet (inlet temperature 225°C) using Hamilton gas-tight syringes. The column oven was kept at 165°C or 180°C. Fatty acid methyl esters were detected by a flame ionization detector. The detector operated at 225°C with a hydrogen/compressed air flame. The carrier gas was oxygen-free N_2 flowing at 35-40 ml per minute.

Esters were identified on the basis of relative retention times. The relative amounts of each fatty acid methyl ester in a sample were obtained by integrating the chromatographic peaks recorded on a chart recorder. Peak integration was done using a fixed-arm planimeter (Hruden) or a MOP III Digital Analyzer (Carl Zeiss (Canada) Ltd., Don Mills, ON). In later work, the chromatograms were integrated automatically by a Hewlett Packard 3390A digital integrator. In most cases, duplicate samples were analyzed and average values calculated.

E. Phosphorus Determination

The phosphorus content of lipid samples containing large amounts of phosphorus (5-50µg) was determined by a modification of the procedure of Fiske and Subbarow (1925) and Dawson (1960). Lipid samples dried from organic solvent or lipid spots scraped from the tlc plates were digested by heating with 0.7 ml of 70% (w/v) perchloric acid for 10 to 20 minutes in 22 X 175 mm Pyrex culture tubes on a digestion apparatus (American Instrument Company, Silver Spring, MA, U.S.A.). Samples were shaken and reheated for several minutes to ensure digestion of material adhered to the sides of the tubes. Reagent blanks and standards containing 40.0µg phosphorus were not heated. Volumes were adjusted to 9.0 ml with distilled water and 0.5-ml of ammonium molybdate (5%, w/v) was added with mixing. A 0.5-ml aliquot of aminonaphtholsulfonic acid (0.25%, w/v) was added to each tube,

the contents were mixed, and the blue color developed at room temperature for 30 minutes. If samples contained gel from tlc plates, the gel was removed by centrifugation in a benchtop centrifuge. The absorbence at 660 nm was measured against distilled water.

The procedure used for samples containing less than 5µg phosphorus was a modification of the method of Bartlet (1959). Samples were digested with 1.0-ml of 70% perchloric acid and the volumes made up to 9.0-ml with distilled water. A standard containing 4.0µg phosphorus was used. After addition of ammonium molybdate and aminonaphtholsulfonic acid, the color was developed by heating in a boiling water bath for ten minutes. Samples were cooled and the absorbance at 815 nm was measured.

F. Purity of Synthetic Lecithins

Lecithin samples (containing approximately 40µg) phosphorus were dissolved in chloroform and applied to tlc plates as single spots. The plates were developed in the phospholipid solvent described above. Spots were visualized using one of the sprays described above. With most samples spots were scraped from tlc plates for phosphorus determination and fatty acid analysis.

Mixed-acid lecithins were also analyzed for positional purity (extent of acyl group migration). A small sample (5-10µg) of lecithin was dissolved in 375µl of

chloroform:methanol:diethyl ether, 5:10:85 (v/v/v). Phospholipase A_2 solution (0.12 mg Crotalus adamanteus venom in 10 µl of 10mM $CaCl_2$) was added and the mixture was shaken vigorously for 90 minutes. Further aliquots of enzyme solution were added at 10, 30 and 60 minutes. Ethanol (300 µl) was added to the mixture at 90 minutes and the solvents were removed in a stream of N_2 gas at 30-40°C. The residue was taken up in 100 µl of chloroform:methanol (2:1 by volume). Two tlc plates were activated and a 50-µl sample was applied to each plate. One plate was developed in the phospholipid solvent; the other was developed in the solvent system for neutral lipids. Plates were stained with dichlorofluorescein. Lecithin and lysolecithin spots were scraped from the phospholipid plate and fatty acid spots from the neutral lipid plate. These were transmethylated and the fatty acid methyl esters were analyzed by glc. Corresponding spots were taken from blank lanes on the same tlc plates for comparison. The fatty acid composition of the lysolecithin and the free fatty acid spots gives the fatty acid distribution at the sn-1 and sn-2 positions of the mixed-acid lecithin. A sample of the lecithin was also applied to the phospholipid plate and the fatty acid composition of the lecithin spot was compared with that of the lecithin taken from solution. Significant disagreement between the compositions of these two samples would indicate the presence of a non-lecithin, fatty acid containing contaminant (eg. fatty acid, anhydride, imidazolide, fatty acid salt of the catalyst or

lysolecithin).

II-6 Differential Scanning Calorimetry (dsc)

A. Preparation of Dispersions

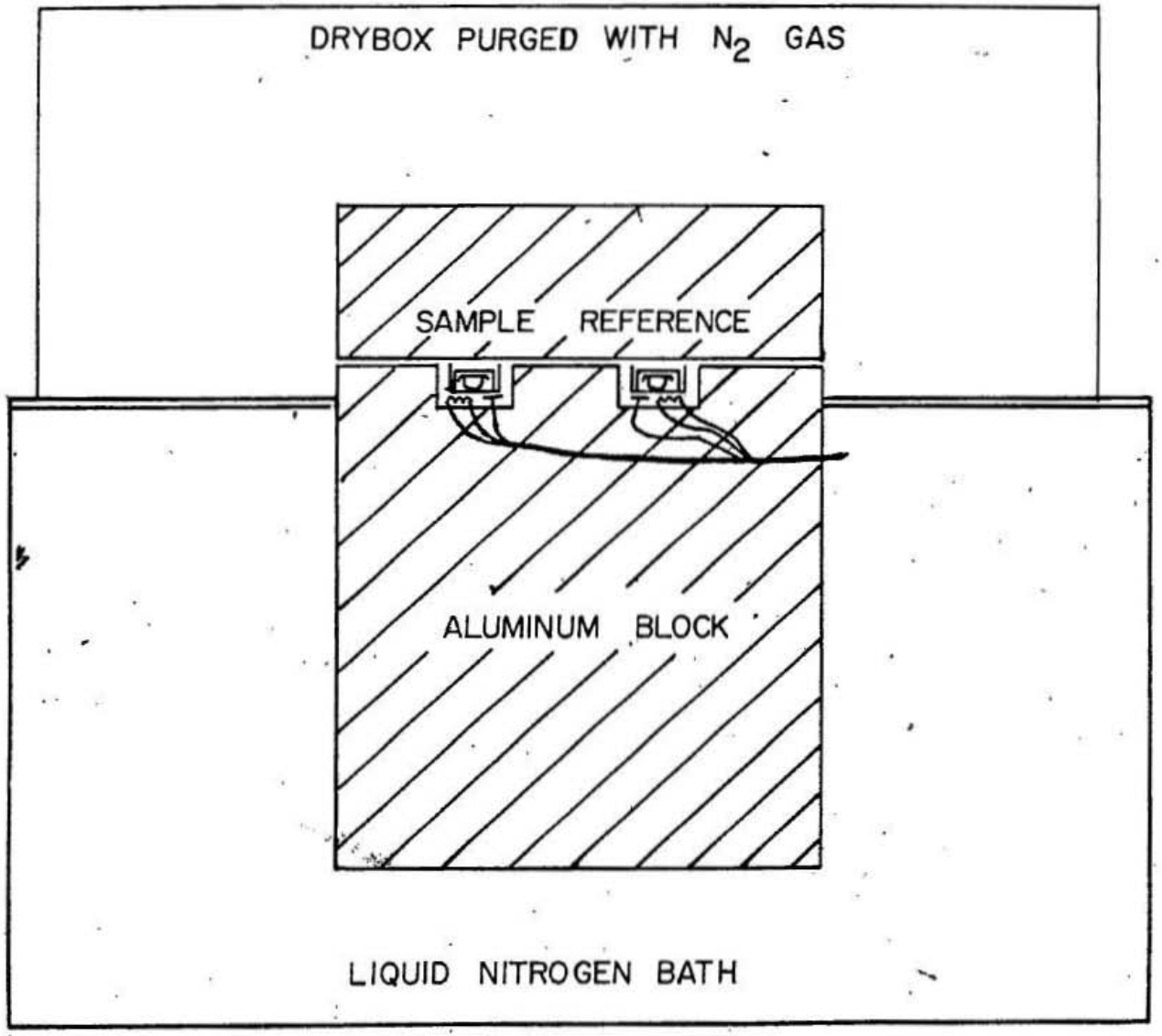
A lecithin sample (4-10 mg) in chloroform solution was transferred to a one dram glass vial with foil lined cap. If the sample contained two components (either two different lecithins or lecithin with cholesterol), the two components in chloroform solution were mixed in a one dram vial. The chloroform was removed in a stream of N_2 gas at 30°C. The dried sample was evacuated over phosphorus pentoxide for 16 hours. The sample was dispersed in water (distilled from alkaline potassium permanganate) by vortexing at temperatures greater than ten degrees above the expected transition temperature. The lipid:water ratio was 1:2 (w/w) unless otherwise noted. The dispersion was transferred to an aluminum sample pan (Perkin Elmer Corporation, Norwalk, CT, U.S.A.) and the pan was sealed using a Perkin Elmer press. Sample volume varied from 8 to 15 µl of dispersion.

B. Calorimeter Operation

Figure II-1 shows a simplified view of the Perkin Elmer DSC-2 analyzer head. A large aluminum block holds the sample and reference pan holder. This block is immersed in a liquid nitrogen bath that serves as a heat sink. The sample holder cavities are purged with dry helium gas. The upper part of the block containing the detection head assembly is

Figure II-1

Diagram of the analyzer head assembly of the Perkin Elmer DSC-2.


DRYBOX PURGED WITH N_2 GAS
SAMPLE
REFERENCE
ALUMINUM BLOCK
LIQUID NITROGEN BATH

covered by a dry box. This box is purged with dry N_2 gas.

The sample and reference are heated or cooled at the same rate. Empty sample pans or pans containing appropriate volumes of water are used as reference. As samples are heated or cooled, a zero temperature difference between sample and reference is maintained by the application of additional power to the sample or reference heater. This differential power is recorded as a function of temperature. The calorimeter has electrical adjustments that allow for some compensation for differences in heat capacity between the sample and reference. In this work calorimeter sensitivities of 1-5 $mcal \cdot sec^{-1}$ (full scale) and programming rates of 5 $deg \cdot min^{-1}$ were used. To obtain endotherms of reasonable size at slower heating rates, increased sensitivities would be required leading to more noise.

C. Calibration of dsc

Periodically throughout the course of these studies the calorimeter was calibrated using standards of known phase transition temperature and enthalpy. Hexadecane (gold label from Aldrich Chemical Company or 99+% pure from Sigma) and Indium (Perkin Elmer Copr.) were used as calibration standards. The relationship between the area under the endotherms or exotherms and the enthalpy of transition was computed based on the known enthalpies of these standards. Area to enthalpy factors were established for the various calorimeter sensitivities used in this work.

D. Determination of Transition Temperature and Enthalpy

Three temperature parameters associated with lipid phase transitions are shown in Figure II-2. The T_{max} is the temperature of maximum excess heat capacity. For sharp transitions the point of intersection of a tangent drawn on the leading edge of the transition with the extrapolated baseline is usually take as the transition temperature (T_c). If transitions are broad and ill-defined, the point of departure of the excess heat capacity line from the extrapolated baseline was used as an estimate of T_c.

The area under the endo- or exotherm was obtained using a fixed-arm planimeter (Hruden) or a MOP III digital analyzer (Carl Zeiss) and the heat associated with the transition (q_{rev}) was computed. The sample pan was opened; the sample was extracted three times with 1.5 ml of chloroform:methanol, 2:1 (by volume) and the amount of lipid phosphorus was determined as described previously. The transition enthalpy (ΔH) was calculated per mole of lipid phosphorus.

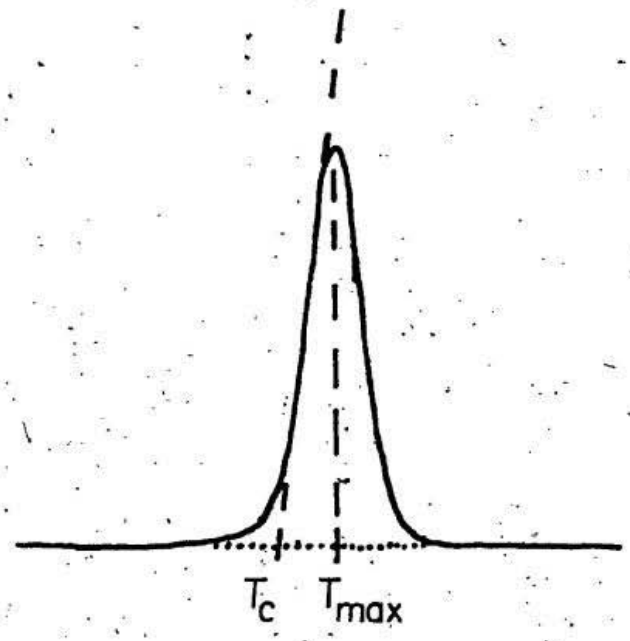
II-7 Analysis of Data

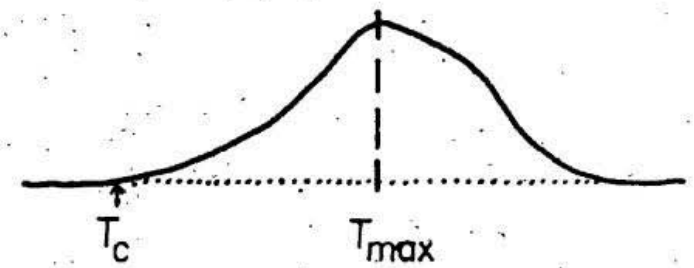
A. Normalization of Endotherms

To facilitate comparisons of the shape and size of endotherms obtained with different lipids or lipid mixtures, endotherms were sometimes normalized. Normalization involved measurement of the deflection (in millimeters) of

Figure II-2

Determination of transition temperatures, T_{max} and T_c, from dsc endotherms.


T_c
T_{max}


T_c
T_{max}

the dsc trace from the extrapolated baseline. These measurements were made at 0.25 degree intervals throughout the transition using a MOP III digital analyzer (Carl Zeiss). The measurements were corrected for calorimeter sensitivity and sample size. The relationship between the measure of the pen deflection from the baseline (d) and the excess heat capacity (C_p^E):

$$C_p^E = \frac{d \cdot S}{D \cdot R \cdot P}$$

where D was the fullscale deflection (in mm), S was the calorimeter sensitivity ($mcal \cdot sec^{-1}$), R was the heating rate ($deg \cdot sec^{-1}$) and P was the amount of lipid phosphorus in the sample (μmol). The normalized values were replotted as excess heat capacity ($cal \cdot deg^{-1} \cdot mol^{-1}$) against temperature (degrees).

B. Deconvolution of Multicomponent Endotherms

The asymmetric endotherms obtained with lecithin-cholesterol dispersions were resolved into two components using a computer program kindly provided by Dr. C. H. Spink. A copy of this program is given in Appendix A. The analysis is based on the assumption that the endotherms result from two superimposed simple two-state transitions. Initial estimates of the maximum excess heat capacity, the T_{max} and the width at half height ($\Delta T_{\frac{1}{2}}$) were obtained from the normalized endotherm by visual inspection and were used to compute estimates of the calorimetric enthalpy (ΔH_{cal}) and the

van't Hoff enthalpy (ΔH_{vH}). Estimates of the two components of the endotherms were then calculated from the van't Hoff enthalpies using an equation of Privalov and Khechinashvili (1974). These components were then summed to give a composite endotherm and the standard deviation of the residuals between this composite endotherm and the normalized DSC endotherm was computed. The T_{max} and the maximum excess heat capacity of the two components of the endotherm were adjusted in an iterative procedure until the difference between the composite endotherm obtained by summing the two components and the experimental data was sufficiently small as judged by the operator (typically the standard deviations of the residuals obtained were 8 to 84 cal·mol^{-1}·deg-1).

C. Construction of Phase Diagrams

The transition temperatures obtained with mixtures of lecithins were used to construct phase diagrams. Temperatures estimated from the intersection of the tangent to the leading edge with the baseline were used for endotherms with sharp onsets. For broad endotherms the baseline departure temperatures were used. Solidus curves were constructed from temperatures obtained on heating scans the liquidus curves were constructed from cooling scan temperatures. All temperatures were corrected for the finite width of the transitions of pure lecithins using the procedure described by Mabrey and Sturtevant (1976). This procedure involves addition to the solidus temperature of the sum of the pro-

ducts of the $\Delta T_{\frac{1}{2}}$ values of each lipid component and the mole fraction for each of the two components of the mixture. Similarly, this amount was subtracted from the liquidus temperature.

D. Theoretical Curves for Ideal Mixing

The solidus and liquidus curves expected if mixing were ideal in both gel and liquid-crystal were generated as described by Mabrey and Sturtevant (1976). The computer programs used to generate these curves is given in Appendix A.

E. Estimates of Non-Ideality of Mixing

Four methods were used in the course of this work to obtain estimates of the excess energy of interaction of unlike molecules in binary lipid mixtures. These were based on Regular Solution Theory (Hildebrand and Scott, 1964) as described for model membranes by Lee (1977).

Method I: If one assumes that the binary mixtures behave as regular solutions and that the two components are miscible in both the gel and the liquid crystal, then estimates of the excess interaction energies in each state are described by the equations (Lee, 1977):

$$\ln\left(\frac{X_A^{liq}}{X_A^{sol}}\right) + \frac{\rho_o^{liq}\left(1-X_A^{liq}\right)^2 - \rho_o^{sol}\left(1-X_A^{sol}\right)^2}{RT} = \frac{H_A}{R}\left(\frac{1}{T_A} - \frac{1}{T}\right) \qquad [1]$$

$$\ln\left(\frac{1-X_A^{liq}}{1-X_A^{sol}}\right) + \frac{\rho_o^{liq}\left(X_A^{liq}\right)^2 - \rho_o^{sol}\left(X_A^{sol}\right)^2}{RT} = \frac{H_B}{R}\left(\frac{1}{T_B} - \frac{1}{T}\right) \qquad [2]$$

where T_A and T_B are the transition temperatures of the pure lecithins, ΔH_A and ΔH_B are the transition enthalpies of the pure lecithins, X_A^{LIQ} and X_A^{SOL} are the mole fractions of the high temperature component in the liquidus and solidus phases respectively at temperature T (degrees Kelvin), and ρ_o^{LIQ} and ρ_o^{SOL} are the excess interaction energies for the two lecithins in the liquidus and solidus phases respectively. In this method Equations [1] and [2] are fitted iteratively to the experimental data until the ρ_o converge (i.e. ρ_o^{LIQ} from Equation [1] does not differ from ρ_o^{LIQ} obtained by fitting Equation [2]). These ρ_o values are unique solutions for the two equations that fit the data. The computer program DPENLN from University of Waterloo Computing Center was used for this procedure.

Method II: In Method II, estimates of ρ_o^{LIQ} and ρ_o^{SOL} were obtained by simultaneous solution of Equations [1] and

[2] at various temperatures between T_B and T_A. The computer program is in Appendix A.

Method III: In mixtures where the shape of the solidus curve suggested extensive immiscibility of the two components in the gel, the data were fitted to an equation from Lee (1978) for non-ideal mixing in the liquid crystal:

$$\frac{\rho_o^{liq}\left(1-X_A^{liq}\right)^2}{H_A} = \frac{T_\cdot}{T_{ideal}} - 1 \qquad [3]$$

where T_{ideal} is the temperature expected if mixing was ideal.

Method IV: This method for estimating ρ_o is similar to Method I in that the two equations are fitted to the entire phase diagram. Using a chosen pair of ρ_o values, pairs of X_A^{LIQ} and X_A^{SOL} values were allowed to vary until the temperatures estimated from Equations [1] and [2] were nearly equal ($\pm$0.2 degrees). This was done for a series of X_A^{LIQ} and X_A^{SOL} pairs. The computer X_A^{LIQ} and X_A^{SOL} values were plotted against the computed T and the resulting curves were compared to the experimental data. Small changes were made in ρ_o^{LIQ} and ρ_o^{SOL} and this procedure was repeated until the mean square residuals were minimized. The computer program is in Appendix A.

III RESULTS

III-1 Synthesis of Lecithins

A. Single-acid Lecithins

The syntheses of single-acid lecithins were carried out to check the effectiveness of the synthetic procedures, to prepare single-acid lecithins containing arachidate chains that were not available from commercial sources, and sometimes to save time when delays in the delivery of commercial samples occurred. Table III-1 summarizes the results from syntheses of these single-acid lecithins. Overall yields, based on the amount of GPC used, were from 15 to 74% in these syntheses. All the preparations were analyzed by tlc using the solvent used to separate phospholipids. After staining the tlc plates with I_2 vapor a large chromatographic spot was observed for each preparation. These spots were attributed to 1,2-diacyl-sn-glycero-3-phosphorylcholine (1,2-lecithin) based on a comparison of their mobility with that of a 1,2-lecithin standard. On some of these tlc plates a small spot with slightly greater mobility than the large spot was also observed. This spot was attributed to 1,3-diacyl-sn-glycero-2-phosphorycholine (1,3-lecithin). Phosphorus was determined in the silica gel scraped from the areas of the tlc plates where the spots were observed. The 1,3-lecithin spots accounted for less than two percent of the total lipid phosphorus.

TABLE III-1

Summary of Synthesis of Single-Acid Lecithins

Preparation	Lecithin	Acylation Procedure
1	DOPC	Keough and Davis (1979)
2	DSPC	Keough and Davis (1979)
3	DOPC	Keough and Davis (1979)
4	DSPC	Keough and Davis (1979)
5	DSPC	Cubero-Robles and van den Berg (1969)
6	DOPC	Keough and Davis (1979)
7	DSPC	Keough and Davis (1979)
8	DSPC	Keough and Davis (1979)
9	DOPC	Gupta et al. (1977)
10	DAPC	Gupta et al. (1977)

*Some of these preparations also showed a minor spot on the tlc plates. This was shown to contain $\leq 2\%$ of the lipid phosphorus.

TABLE III-1 (continued)

†	Acylation Temp.	Acylation Time	Purification Procedure
1	75°C	24 hr.	silicic acid column chromatography
2	95°C	24 hr.	silicic acid column chromatography two acetone precipitations
3	65°C	24 hr.	silicic acid column chromatography
4	85°C	36 hr.	silicic acid column chromatography two acetone precipitations
5	90°C	25 hr.	silicic acid column chromatography and two acetone precipitations
6	75°C	24 hr.	silicic acid column chromatography
7	85°C	24 hr.	Lobar column chromatography and two acetone precipitations
8	95°C	24 hr.	silicic acid column chromatography and two acetone precipitations
9	25°C	36 hr.	CM-52 chromatography
10	25°C	48 hr.	CM-52 chromatography and three acetone precipitations

†refers to Preparation number.

TABLE III-1 (continued)

†	Yield	Analysis*
1	1.4g (49%)	single spot seen on tlc plate; greater than 99% oleic acid by glc.
2	1.6g (74%)	single spot on tlc; greater than 99% stearate by glc.
3	2.0g (49%)	single spot on tlc plate
4	1.5g (15%)	single spot on tlc plate
5	4.7g (40%)	single spot on tlc plate
6	1.3g (17%)	single spot on tlc plate
7	1.9g (22%)	single spot on tlc plate
8	11.4g (52%)	single spot on tlc plate
9	765mg (18%)	single spot on tlc plate
10	567mg (24%)	single spot on tlc plate

†refer to Preparation number.

B. Mixed-acid Lecithins

At the start of this work there were no mixed-acid lecithins available from commercial sources. Recently a limited number of mixed-acid lecithins have become available. With the exception of 1-oleoyl-2-palmitoyl lecithin (OPPC), which was purchased from a commercial supplier, the mixed-acid lecithins used in this work were synthesized from appropriate single-acid lecithins. The positional purity of mixed-acid lecithins containing saturated acyl chains affected their thermotropic properties (Keough and Davis, 1979). Therefore, part of this work was devoted to establishing synthetic procedures that resulted in mixed-acid lecithins containing only small amounts of the reversed positional isomers. The results from the syntheses of mixed-acid lecithins using different synthetic procedures are summarized in Table III-2.

i. Preparation of Lysolecithin-Phospholipase A_2 Hydrolysis

Two different procedures were used to prepare lysolecithin by phospholipase A_2 hydrolysis of the single-acid lecithins. Lysolecithin preparations made using either of the two procedures did not exhibit a lecithin spot on tlc plates stained with I_2 vapor. In later preparations, lysolecithin spots and the silica gel, from the areas where lecithin spots would be expected, were scraped from the tlc plates and phosphorus determinations were carried out as

TABLE III-2

Summary of Synthesis of Mixed-Acid Lecithins

Preparation	Lipid	Hydrolysis Procedure	Hydrolysis Time
1	OSPC	Keough and Davis (1979)	90 min.
2	SOPC	Keough and Davis (1979)	90 min.
3	OSPC	Keough and Davis (1979)	90 min.
4	SOPC	Keough and Davis (1979)	120 min.
5	OSPC	Keough and Davis (1979)	90 min.
6	SOPC	Keough and Davis (1979)	110 min.
7	SOPC	Keough and Davis (1979)	120 min.
8	SOPC	Keough and Davis (1979)	120 min.
9	SOPC	Keough and Davis (1979)	120 min.
10	OSPC	Keough and Davis (1979)	100 min.
11	OSPC	Chakrabarti and Khorana (1975)	90 min.
12	SOPC	Chakrabarti and Khorana (1975)	90 min.
13	OSPC	Keough and Davis (1979)	120 min.
14	OSPC	Keough and Davis (1979)	100 min.
15	OSPC	Keough and Davis (1979)	130 min.
16	OAPC	Keough and Davis (1979)	100 min.
17	AOPC	Keough and Davis (1979)	130 min.
18	AOPC	Keough and Davis (1979)	100 min.

continued

TABLE III-2 (continued)

†	Acylation Procedure	Acylation Temp.	Acylation Time	Conversion LysoPC to PC
1	Keough and Davis (1979)	75°C	6.5 hr.	N.D.
2	Keough and Davis (1979)	75°C	6.0 hr.	N.D.
3	Keough and Davis (1979)	85°C	6.0 hr.	N.D.
4	Keough and Davis (1979)	65°C	3.5 hr.	N.D.
5	Keough and Davis (1979)	85°C	6.5 hr.	N.D.
6	Keough and Davis (1979)	75°C	6.0 hr.	N.D.
7	Warner and Benson (1977)	25°C	5 min.	0%
8	Warner and Benson (1977)	25°C	15 min.	N.D.
9	Warner and Benson (1977)	25°C	15 min.	N.D.
10	Warner and Benson (1977)	25°C	15 min.	N.D.
11	Gupta et al. (1977) using free lysoPC	25°C	30.0 hr.	N.D.
12	Gupta et al. (1977) using LysoPC·$CdCl_2$	25°C	33.0 hr.	97.2%
13	Gupta et al. (1977) using free LysoPC	25°C	27.5 hr.	N.D.
14	Gupta et al. (1977) using free LysoPC	25°C	30.0 hr.	N.D.
15	Gupta et al. (1977) using free LysoPC	25°C	36.0 hr.	N.D.
16	Gupta et al. (1977) using free LysoPC	25°C	34.0 hr.	92%
17	Gupta et al. (1977) using free LysoPC	25°C	48.0 hr.	76%
18	Gupta et al. (1977) using free LysoPC	25°C	40.0 hr.	74%

†refers to preparation number.

- continued

TABLE III-2 (continued)

†	Purification Procedures	Fatty Acid Comp. of neat PC product	Fatty Acid Comp. of PC spot from tlc
1	silicic acid chromatography and two acetone ppt.	N.D.	47mol% 18:1; 51mol% 18:0; 2mol% 16:0
2	silicic acid chromatography and two acetone ppt.	51mol% 18:0; 48.5mol% 18:1	50.5mol% 18:0 49.5mol% 18:1
3	silicic acid chromatography and two acetone ppt.	N.D.	50.8mol% 18:1 49.2mol% 18:0
4	silicic acid chromatography and two acetone ppt.	49.8mol% 18:0 50.2mol% 18:1	50.8mol% 18:0 49.2mol% 18:1
5	silicic acid chromatography and two acetone ppt.	50.8mol% 18:1 49.2mol% 18:0	N.D.
6	silicic acid chromatography and two acetone ppt.	50.1mol% 18:0 49.9mol% 18:1	50.6mol% 18:0 49.4mol% 18:1
7			
8	silicic acid chromatography	49.2mol% 18:0 50.8mol% 18:1	N.D.
9	preparative tlc.	N.D.	N.D.
10	silicic acid chromatography and two acetone ppt.	N.D.	49.2mol% 18:1 50.8mol% 18:0
11	silicic acid chromatography and two acetone ppt.	N.D.	48.9mol% 18:1 51.5mol% 18:0
12	silicic acid chromatography and two acetone ppt.	N.D.	50.2mol% 18:0 49.8mol% 18:1
13	CM-52 chromatography and two other ppt.	50.0mol% 18:1 50.0mol% 18:0	N.D.
14	CM-52 chromatography and two other ppt.	50.3mol% 18:1 49.7mol% 18:0	50.5mol% 18:1 49.5mol% 18:0
15	CM-52 chromatography and three acetone ppt.	N.D.	54mol% 18:1 46mol% 18:0
16	CM-52 chromatography and three acetone ppt.	51.5mol% 18:1 48.5mol% 20:0	49.5mol% 18:1 50.5mol% 20:0
17	CM-52 chromatography and three acetone ppt.	49.6mol% 20:0 50.4mol% 18:1	52.5mol% 20:0 47.5mol% 18:1
18	CM-52 chromatography and three acetone ppt.	49.0mol% 20:0 51.0mol% 18:1	51.4mol% 20:0 48.6mol% 18:1

†refers to preparation number.

continued

TABLE III-2 (continued)

†	Fatty Acid Comp. of lyso spot from tlc	Fatty Acid Comp. of fatty acid spot from tlc	Acyl Migration	Yield
1	80mol% 18:1; 17mol% 18:0; 3mol% 16:0	13mol% 18:1; 87mol% 18:0	17%	N.D.
2	74mol% 18:0 26mol% 18:1	38mol% 18:0 62mol% 18:1	26%	250mg (42%)
3	86.3mol% 18:1 13.7mol% 18:0	30mol% 18:1 70mol% 18:0	14%	N.D.
4	85.3mol% 18:0 14.7mol% 18:1	15.5mol% 18:0 84:5mol% 18:1	15%	600mg (20%)
5	84mol% 18:1 16mol% 18:0	19mol% 18:1 81mol% 18:0	16%	N.D.
6	82.9mol% 18:0 17.1mol% 18:1	23mol% 18:0 77mol% 18:1	17%	560mg (28%)
7				
8	90.4mol% 18:0 9.6mol% 18:0	18mol% 18:0 82mol% 18:1	10%	20mg (10%)
9	N.D.	N.D.	N.D.	8mg (4%)
10	75mol% 18:1 25mol% 18:0	26mol% 18:1 74mol% 18:0	25%	150mg (10%)
11	81.7mol% 18:1 18.3mol% 18:0	22mol% 18:1 78mol% 18:0	18%	700mg (41%)
12	94.7mol% 18:0 5.3mol% 18:1	6.7mol% 18:0 93.3mol% 18:1	5%	586mg (59%)
13	>99mol% 18:1 < 1mol% 18:0	1.4mol% 18:1 98.6mol% 18:0	<1%	325mg (65%)
14	>99mol% 18:1 < 1mol% 18:0	<1mol% 18:1 >99mol% 18:0	<1%	164mg (66%)
15	>99mol% 18:1 < 1mol% 18:0	3.7mol% 18:1 96.3mol% 18:0	<1%	810mg (81%)
16	>99mol% 18:1 < 1mol% 20:0	7mol% 18:1 93mol% 20:0	<1%	420mg (84%)
17	94mol% 20:0 6mol% 18:1	91mol% 20:0 9mol% 18:1	6%	390mg (78%)
18	>99mol% 20:0 < 1mol% 18:1	<1mol% 20:0 >99mol% 18:1	<1%	340mg (81%)

†refers to preparation number.

described before. The lysolecithin spots contained >99% of the total lipid phosphorus. The nearly equimolar fatty acid composition of all the mixed-acid lecithin preparations was a further indication that only trace amounts (if any) of the single-acid lecithins were present in the lysolecithin preparations.

Following the discovery that, under certain conditions, lysolecithin could breakdown to form GPC (see Section III-1Biv), the silica gel from the areas of the tlc plates where the lysolecithin samples were applied (origins) was scraped and the phosphorus content was determined. In all preparations, except those described in Section III-1Biv, less than one percent of the total lipid phosphorus was present at the origins.

ii. Acylation of Lysolecithin and Purification of Mixed-acid Lecithins

The yields obtained with the different acylation and purification procedures varied (Table III-2). A procedure described by Warner and Benson (1977) for the acylation of GPC using fatty acid imidazolides resulted in very poor yields (0 to 10%) when used in my hands to acylate lysolecithin. The modification of the procedure of Cubero-Robles and van den Berg (1969) described by Keough and Davis (1979) for the preparation of saturated mixed-atid lecithins gave intermediate yields of 20 to 42%. A procedure reported by Gupta et al. (1977) resulted in 60 to 84% yield. The

procedure selected after a consideration of a large number of syntheses was: phospholipase A_2 hydrolysis as described by Keough and Davis (1979); acylation of the lysolecithin as described by Gupta et al. (1977); and purification by CM-52 column chromatography (Comfurius and Zwaal, 1977). The major losses in this procedure occurred during the acylation and Rexyn I-300 ion exchange chromatography.

iii. Acyl Group Migration

The extent of acyl migration occurring during the syntheses, as indicated by the amount of the "wrong" positional isomers formed, varied with the different procedures (Table III-2). In the mixed- acid lecithins synthesized using the Warner and Benson procedure (1977) acyl migration was 8 to 25%. Mixed-acid lecithins prepared using the acylation procedure described by Keough and Davis (1979) contained 14 to 26% of the wrong positional isomers. The lowest acyl migration occurred in the syntheses using the acylation method of Gupta et al. (1977). With one exception (preparation 11, Table III-2) all the mixed-acid lecithins prepared by this procedure had less than six percent of the reversed positional isomers, and in four of these preparations no products of acyl migration were detected.

In almost all the analyses of the positional distribution of mixed-acid lecithins the composition of the fatty acids liberated from the sn-2 position of the lecithins indicated greater acyl migration than was indicated by the

fatty acid composition of the lysolecithin. In these preparations the fatty acid compositions of the lecithins before and after purification by tlc were the same within limits of the glc analysis. For this reason the discrepancy between the positional distribution based on the analysis of the hydrolyzed fatty acids and that based on the analysis of the lysolecithin cannot be attributed to contamination of the mixed-acid lecithin by free fatty acids or fatty acid anhydrides. Some of the single-acid lecithins contained small amounts of 1,3-lecithin and small amounts of 1,3-lecithins were found in some of the mixed-acid lecithins. If the 1,3-lecithins in the mixed-acid lecithin preparations were single-acid 1,3-lecithins that remained unhydrolyzed during the phospholipase A_2 hydrolysis step in the synthetic procedures, this might account for the discrepancies in the positional analyses. To investigate this possibility, the 1,3-lecithin and the 1,2-lecithin in one mixed-acid lecithin preparation were separated by tlc, and a positional analysis was carried out on both the 1,3-lecithin and the 1,2-lecithin. The results of this analysis (Table III-3) showed that the positional distribution of the 1,3-lecithin was almost identical to that of the 1,2-lecithin and no indication of the presence of single-acid 1,3-lecithin was found. As will be discussed below (Section IV-1B) these discrepancies may arise because of acyl migration occurring during the hydrolysis by phospholipase

TABLE III-3

Positional analysis of 1,2-Lecithin and 1,3-Lecithin separated by thin layer chromatography.

	1,2-Lecithin	1,3-Lecithin
Lyso formed by phospholipase A_2 hydrolysis of preparation #4 (Table III-2)	16mol% Oleate 84mol% Stearate	15mol% Oleate 85mol% Stearate
Fatty acids liberated by hydrolysis of preparation #4 (Table III-2)	85mol% Oleate 15mol% Stearate	84mol% Oleate 16mol% Stearate
Acyl migration	16%	15%

A_2 (Pluckthun and Dennis, 1982).

iv. Breakdown of Lyso-oleoyl Lecithin

Early attempts to synthesize OSPC using the cadmium chloride adduct of lyso-oleoyl lecithin prepared by the procedure of Chakrabarti and Khorana (1975) and the acylation procedure of Gupta et al. (1977) resulted in lecithin products containing excess sterate chains (Table III-4). Some of the final products were almost pure DSPC as determined by the tlc and glc analysis. In one case the product was analyzed by dsc and it had the same transition temperature as pure DSPC. These results suggested the possibility that the lyso-oleoyl lecithin was breaking down to GPC before acylation. As can be seen from the detailed analysis of preparations 7 and 8 (Table III-4), a substantial amount of the lipid phosphorus in these preparations was contained in a component that had no mobility as tlc plates developed in the solvent system for the separation of phospholipids. GPC also showed no mobility in this solvent system. These lysolecithin preparations had a component that was insoluble in chloroform, in chloroform:methanol, 1:1 (by volume) or in ethanol. The precipitate was collected by centrifugation and dissolved in methanol:water, 7:3 (by volume). To ensure that the breakdown product was GPC, samples of both the supernatant and the precipitate from the lysolecithin preparation were applied to another tlc plate. A variety of other potential lyso-oleoyl lecithin degradation products

TABLE III-4

Summary of data on the breakdown of Lyso-oleoyl lecithin.

Lyso-oleoyl PC preparation	Borate Buffer used in hydrolysis?	Exposed to Ethanol?	Observations
1	+	+	After acylation with stearoyl anhydride (Gupta et al., 1977) a glc analysis showed that the lecithin product contained 68mol% stearate and 32mol% oleate.
2,3,5,6	+	+	After acylation with stearoyl anhydride (Gupta et al., 1977) a glc analysis showed that the lecithin product contained > 99mol% stearate.
4	+	+	A small scale (50mg DOPC hydrolyzed) preparation. After acylation with stearoyl anhydride (Gupta et al., 1977) a glc analysis showed that the lecithin product contained 51mol% stearate and 49mol% oleate. The yield was very poor (10%).
7	+	+	This preparation of lyso-oleoyl PC was not acylated. The phosphorus content in the spots observed on tlc plates of this preparation before and after a 2 hour exposure to ethanol was:

	before ethanol	after ethanol
origin	0.4 µg	38.3 µg
lyso spot	15.7 µg	0.7 µg
lecithin spot	0.4 µg	1.1 µg

TABLE III-4 (continued)

Lyso-oleoyl PC preparation	Borate Buffer used in hydrolysis?	Exposed to Ethanol?	Observations
8	+	+	This preparation was not acylated. After exposure to ethanol, a sample was applied to a tlc plate and the plate developed in the solvent system for separation of phospholipids. The phosphorus content of the spots on the tlc plate was: origin 51.5 g lyso spot 122.2 g lecithin spot 0.5 g
9	+	+	See Table III-5 for detailed analysis.

were also applied to this plate and the chromatogram was developed in methanol:water, 7:3 by volume (Chada, 1970). A sketch of this plate after staining with I_2 vapor and then with modified Dittmer-Lester reagent (Ryu and MacCoss, 1979) is shown in Figure III-1. The major spot had the same mobility as GPC and the supernatant had components with the mobilities of GPC, oleic acid or ethyl oleate, and some unidentified components. Ethyl oleate and methyl oleate were also detected in the preparations by glc analysis. Lyso-oleoyl lecithin prepared as described by Keough and Davis (1979), was stable and did not break down. It appeared that the phospholipase A_2 hydrolysis in borate buffer (Chakrabarti and Khorana, 1975) predisposed the lyso-oleoyl lecithin to subsequent breakdown on exposure to ethanol. To check this possibility, a preparation of lyso-oleoyl lecithin was made using the procedure of Keough and Davis (1979). Table III-5 shows that exposure of this material to a variety of solvents, including ethanol, did not cause breakdown. The lyso-oleoyl lecithin was then treated with the borate buffered phospholipase A_2 described by Chakrabarti and Khorana (1975). Exposure of this material to chloroform:methanol, 1:1 (by volume) caused the formation of some GPC (<7%) but exposure to ethanol resulted in breakdown of almost all of the lysolecithin. Since ethyl esters of fatty acids could be formed in acidic or alkaline ethanol, the pH of the ethanol was checked and found to be pH7.4 when measured with a combination glass electrode and a

Figure III-1

Sketch of tlc plate of lyso-oleoyl lecithin that was prepared using borate buffered phospholipase A_2 (Chakrabarti and Khorana, 1975) and exposed to ethanol. Plate was developed in Methanol:Water 7:3 (by volume, Chada, 1970) and stained with I_2 vapor and then with modified Dittmer-Lester reagent (Ryu and MacCoss, 1979). * indicates the chromatographic spots that were positive for phosphorus.

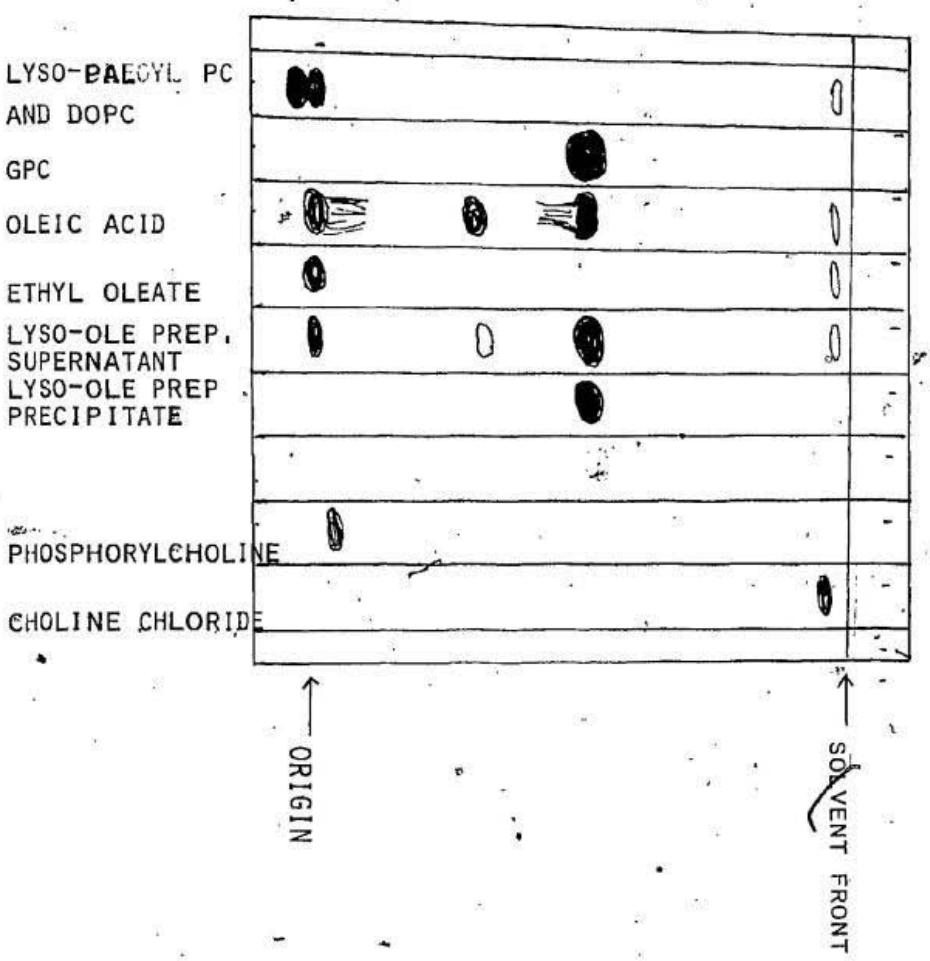

LYSO-PALCYL PC
AND DOPC
GPC
OLEIC ACID
ETHYL OLEATE
LYSO-OLE PREP.
SUPERNATANT
LYSO-OLE PREP
PRECIPITATE
PHOSPHORYLCHOLINE
CHOLINE CHLORIDE
ORIGIN
SOLVENT FRONT

TABLE III-5

Phosphorus analysis of spots from tlc plates of lyso-ole PC prepared according to Keough & Davis was exposed to different solvents for 16 hours and run on tlc.

	SOLVENT				
	Chloroform	Methanol	Ethanol	Chloroform/ Methanol	Ethanol/ Ether
P at origin	0.0 µg	0.0 µg	0.0 µg	0.0 µg	0.0 µg
P in lyso	28.4	33.2	33.2	33.7	30.5
P in PC	0.0	0.0	0.0	0.0	0.0
P applied	28.9	31.0	34.2	34.2	35.8

The lyso from above was then treated according to the method of Chakrabarti in borate buffer and exposed to solvents.

	SOLVENT	
	Chloroform/ Methanol	Ethanol
P at origin	0.9 µg	32.4 µg
P in lyso	13.1	2.6
P applied	13.6	38.2

Radiometer pH meter. Redistilled ethanol also had a pH of 7.4. Later attempts to reproduce the breakdown of lyso-oleoyl lecithin were unsuccessful. In these attempts a new batch of ethanol was used and this suggests that a contaminant in the ethanol used in the earlier work may have been responsible for the breakdown. It is noteworthy that no ethanol-induced breakdown of lysolecithin was observed in one small scale preparation of lyso-oleoyl lecithin prepared using the procedure of Chakrabarti and Khorana (1975). Also, lyso-palmitoyl lecithin and lyso-stearoyl lecithin (Coolbear and Keough, unpublished) prepared using this procedure were stable in ethanol. Thus, whatever the mechanism involved in the breakdown, it would appear to require the presence of an unsaturated acyl chain.

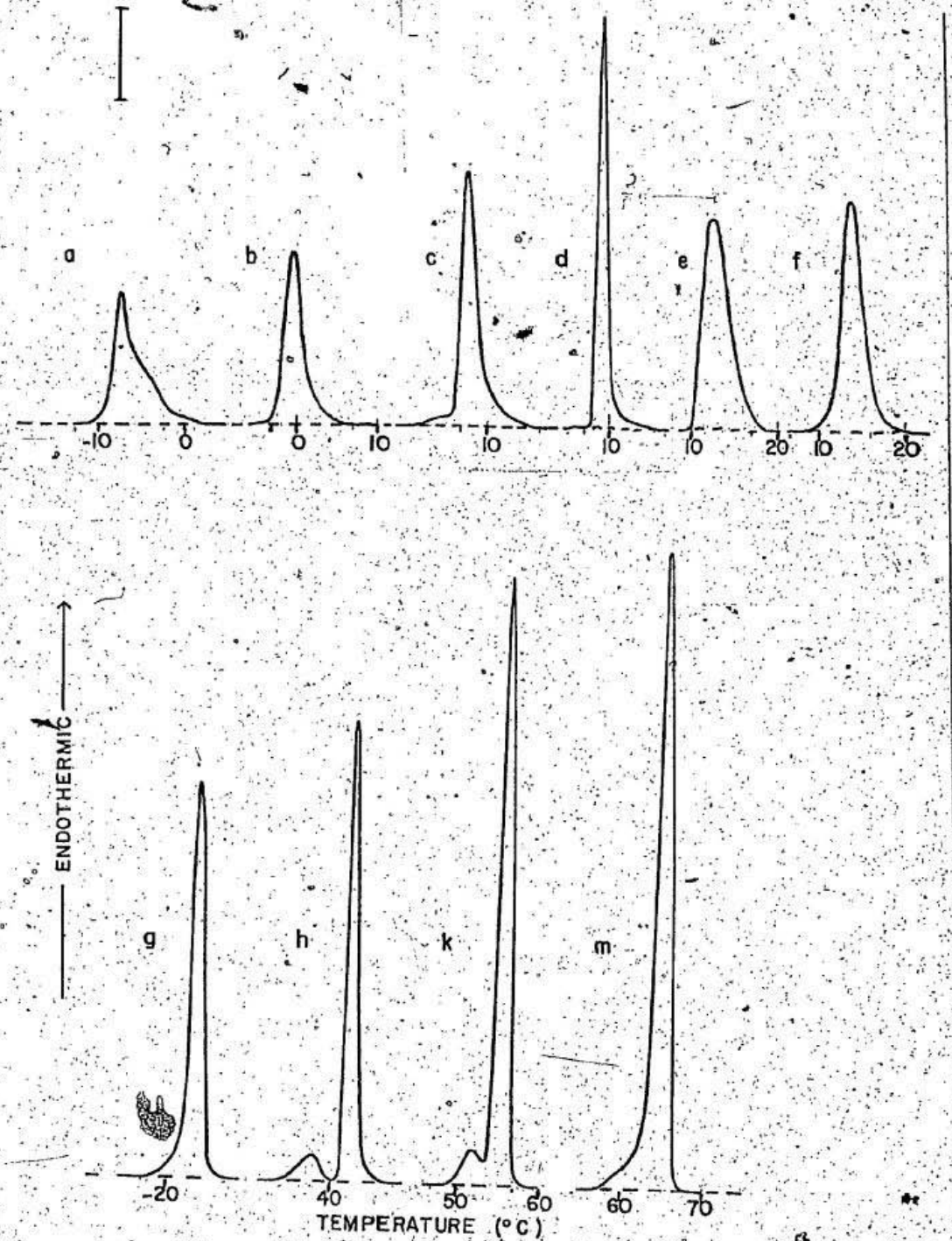
III-2 Thermotropic Behavior of Mixed-acid and Single-acid Lecithins

A. The Shapes of the Endotherms

Typical normalized dsc endotherms obtained with mixed-acid lecithins containing oleate and either palmitate, stearate or arachidate chains are shown in Figure III-2. The endotherms obtained with the "parent" single-acid lecithins DOPC, DPPC, DSPC and DAPC are also shown. Several of the mixed-acid lecithin (OPPC, POPC, OSPC and SOPC) have transitions in the temperature range of the ice-water transition. Dispersions of these lecithins were supercooled to 261 to 265K (-12° to -8°C) and heating scans were begun at

Figure III-2

Normalized dsc endotherms obtained on heating aqueous dispersions of OPPC (a), POPC (b), SOPC (c), OSPC (d), AOPC (e), OAPC (f), DOPC (g), DPPC (h), DSPC (k), and DAPC (m). The scale in the upper left represents 1.0 $kcal \cdot mol^{-1} \cdot deg^{-1}$.


a
b
c
d
e
f
-10
0
0
10
10
10
10
20
10
20
ENDOTHERMIC
g
h
k
m
-20
40
50
60
60
70
TEMPERATURE (°C)

this temperature. This allowed the transition endotherms to be studied without using ethylene glycol in the dispersions. The endotherms of OPPC dispersions in ethylene-glycol-water, 1:1 (by volume) were investigated. The transition temperature in the presence of ethylene glycol was the same as the temperature in pure water for heating scans but cooling scans had transition temperature in the presence of ethylene glycol that were approximately 4°C higher than in pure water. Moreover the presence of ethylene glycol in the dispersions enhanced the high-temperature asymmetry of OPPC endotherms. The PQPC used in this study was prepared by K. P. Coolbear and B. D. Fleming.

The endotherms obtained on heating the dispersions of the mixed-acid lecithins exhibited asymmetry on the high-temperature side of the transitions. This asymmetry was seen with preparations having no acyl migration (OPPC and OAPC) as well as with preparations containing 18 mol% of the "wrong" positional isomers (SOPC). The unsaturated single-acid lecithin (DOPC) exhibited a slight low-temperature asymmetry. The endotherms shown in Figure III-2 were obtained with the preparations of mixed-acid lecithins having the least acyl migration (see Table III-2). Comparable endotherms were obtained with preparations having greater acyl migration but the endotherms were broader and in some cases the transition enthalpies were different from those of the endotherms shown in Figure III-2 (eg. the Δ H values were 5.3 kcal mol^{-1} and 6.6 kcal mol^{-1} for SOPC preparations

containing 18% and 6% OSPC respectively).

No pretransition endotherms were observed in heating scans of the saturated-unsaturated mixed-acid lecithins or the unsaturated single-acid lecithins. The dispersion of DPPC and DSPC exhibited pretransitions similar to those reported by others and DAPC appeared to have a pretransition endotherm that was almost coincidental with the main transition.

B. Thermotropic Properties

i. Transition Temperatures

The thermotropic properties of the mixed-acid and single-acid lecithins are summarized in Table III-6. The T_c and T_{max} values of the mixed-acid lecithins were intermediate between those of the respective parent single-acid lecithins. In the lecithins studied here, the transition temperatures of both positional isomers in each isomeric pair were closer to the transition temperature of DOPC than to that of the other parent saturated single-acid lecithin.

ii. Transition Widths

The widths of the transition endotherms at half maximum excess heat capacity ($\Delta T_{\frac{1}{2}}$) can provide an indication of the cooperativities of the transitions (narrower transitions reflect larger cooperative units). The $\Delta T_{\frac{1}{2}}$ values obtained with the lecithins studied here are shown in Table III-6.

TABLE III-6

Summary of the Thermotropic Properties of Single-acid and Mixed-acid Lecithins.

LECITHIN	Source	Positional Purity	N	T_c	T_{max}	$T_{1/2}$	H
OPPC	#Sigma	99%	3	-9.3±0.9	-7.9±0.9	2.3±0.5	4.6±0.8
POPC	#BDF	94%	5	-2.6±0.2	-0.8±0.5	2.4±0.3	5.4±0.3
SOPC	†Prep 12	95%	3	6.2±0.4	8.0±0.2	1.9±0.2	6.6±0.4
OSPC	†Prep 14	99%	3	8.1±0.3	10.4±0.4	1.6±0.1	6.4±0.4
AOPC	†Prep 17	94%	3	10.1±0.3	11.9±0.2	2.5±0.6	4.2±0.3
OAPC	†Prep 18	99%	5	14.9±0.3	17.8±0.4	2.9±0.2	6.9±0.9
DSPC	†Prep 8		2	55.3,55.1	56.5,56.5	2.0,2.0	9.1,9.2
DAPC	†Prep 10		3	64.4±0.4	66.2±0.3	1.9±0.3	14.9*
DOPC	†Prep 9		2	-15.7,-15.8	-16.5,-16.5	1.6,1.8	8.2,8.3

*Only one enthalpy determination -- with other samples the sample pans leaked at the high temperature and some dispersion was lost.

†Refers to preparations listed in Tables III-1 and III-2.

#Sigma refers to Sigma Chemical Co., St. Louis, MO, U.S.A.
BDF refers to preparation from B. D. Fleming.

The values range from 1.6 to 2.9°K. The $\Delta T_{\frac{1}{2}}$ values of the isomers in each of the isomeric pairs were not significantly different from each other. There were small but significant differences in $\Delta T_{\frac{1}{2}}$ values between the isomeric pairs containing oleate and palmitate or arachidate chains and the isomeric pair containing oleate and stearate chains, the latter pair having the smallest $\Delta T_{\frac{1}{2}}$ values. The $\Delta T_{\frac{1}{2}}$ values obtained with the dispersions of the single-acid lecithins were similar to those obtained with SOPC and OSPC.

iii. Transition Enthalpies

The transition enthalpies (Δ H) of each of the mixed-acid lecithins were lower than those of any of the single-acid lecithins (Table III-6). No significant differences in the transition enthalpies were seen between OPPC and POPC or between OSPC and SOPC, but the enthalpy of transition of dispersions of AOPC was significantly less than that of OAPC dispersions. The sample to sample variability in the enthalpy values was high for these dispersions.

iv. Cooling Scans

Although the exotherms obtained on cooling of the lecithin dispersions were not analyzed in the detail applied to the heating endotherms, the shapes of the exotherms were commensurate with the heating endotherms. In all cases the temperatures of maximum excess heat capacity (T_{max}) of the liquid-crystalline to gel transitions were slightly lower (1

to 2 K) than the T_{max} values obtained on heating scans. This suggests that these dispersions may have been subject to supercooling or thermal lags, or both.

III-3 Lecithin-Cholesterol Mixtures

The effects of cholesterol on the behavior of single-acid phospholipids containing saturated acyl chains have been studied extensively in both monolayers and bilayers (for a review see Demel and de Kruijff, 1976). Most of the information currently available on cholesterol-lipid interactions has been obtained using mixtures of cholesterol with the saturated single-acid lecithins, DMPC and DPPC. Many biological membranes contain mixed-acid lipids having a saturated acyl chain at the sn-1 position and an unsaturated acyl chain at the sn-2 position. It was, therefore, of considerable importance to determine if the lecithin-cholesterol interactions were different for systems containing single-acid or mixed-acid lipid. It has been suggested (Huang, 1977) that cholesterol might interact differently with different fatty acid positional isomers of mixed-acid lecithins containing both a saturated acyl chain and an unsaturated acyl chain. Furthermore, unusually high cholesterol levels have been observed in biological systems in which the positional distribution of fatty acids in the phospholipids showed greater proportions of the less common 1-unsaturated-2-saturated lipids (Rottem and Markowitz, 1979; Dyatlovitskaya et al., 1974, 1975). Thus, the question

whether positional distribution of fatty acids in phospholipids might influence their interactions with cholesterol was also of some importance.

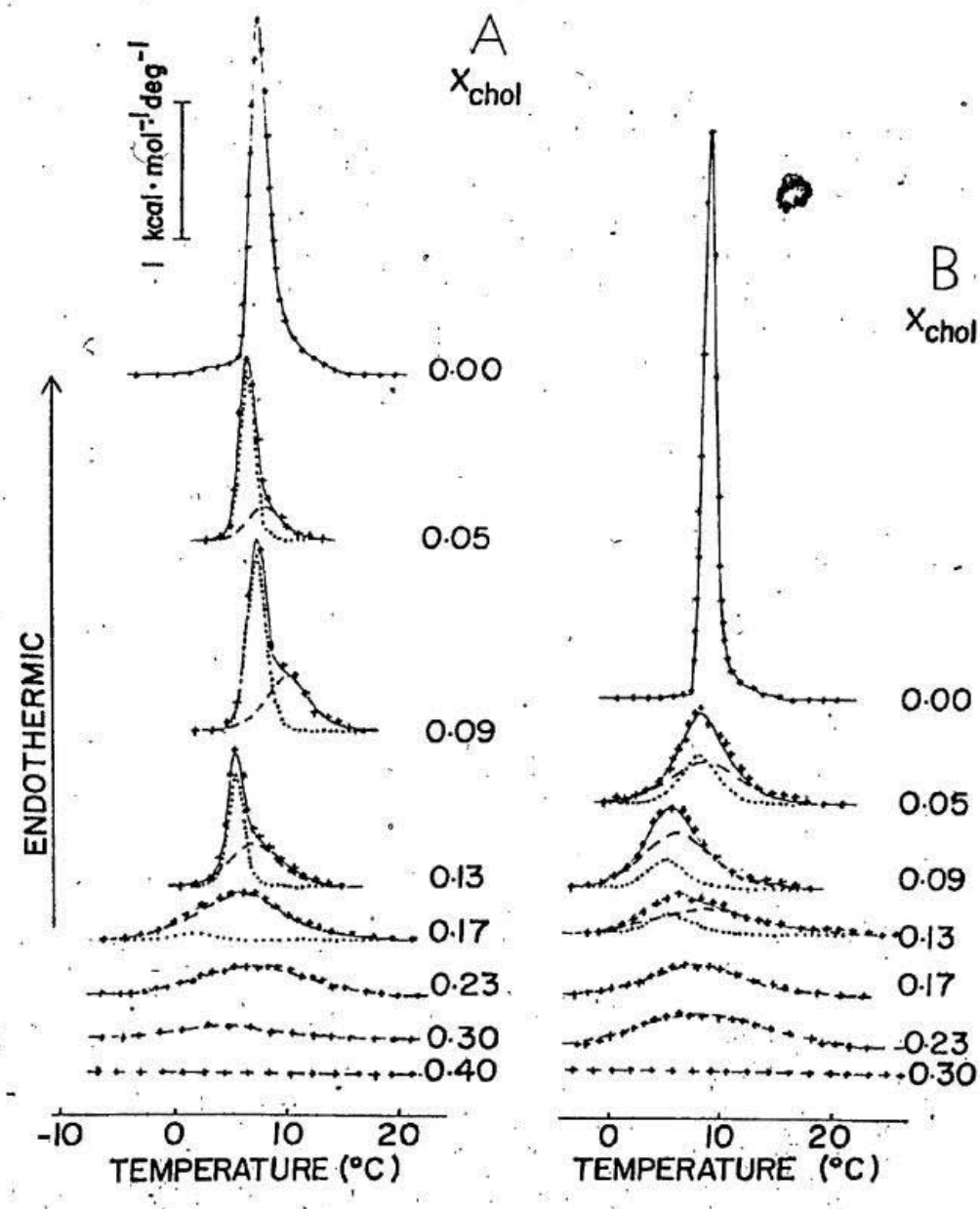
The previous section described the effects on thermotropic behavior, of differences in the acyl chain distribution in saturated-unsaturated mixed-acid lecithins (lecithins containing both a saturated chain and an unsaturated chain). To determine the consequences of changes in the positional distribution of the acyl chains in saturated-unsaturated mixed-acid lecithins on the lecithin-cholesterol interactions, thermograms were obtained for dispersions of mixtures of various mixed-acid lecithins with cholesterol. For comparison, the effects of cholesterol on the respective parent single-acid lecithins were also studied.

A. Effects of Cholesterol on OSPC and SOPC.

Normalized endotherms obtained on heating aqueous dispersions of mixtures of cholesterol with OSPC and with SOPC are shown in Figure III-3. Addition of cholesterol to both lecithins resulted in a decrease in the maximum of the excess heat capacity of the transition endotherms and the appearance of an increased high-temperature asymmetry in the endotherms. Assuming the asymmetric endotherms obtained with the lecithin-cholesterol mixtures consisted of two superimposed endotherms (Estep et al., 1978; Mabrey et al., 1978) those of the mixtures containing from 5 to 17mol% cholesterol were resolved into two components as described

Figure III-3

Normalized dsc endotherms obtained on heating aqueous dispersions of mixtures of cholesterol with SOPC (A) and OSPC (B), and the components of the endotherms. (+) points from the normalized dsc endotherms; (——) composite endotherms, (·····) narrow components of the endotherm, and (– – –) broad components of the endotherms obtained using the computer analysis described in Section II-7E.


A
x_{chol}
1 kcal · mol^{-1} · deg^{-1}
0·00
0·05
0·09
0·13
0·17
0·23
0·30
0·40
ENDOTHERMIC
-10
0
10
20
TEMPERATURE (°C)
B
x_{chol}
0·00
0·05
0·09
0·13
0·17
0·23
0·30
0
10
20
TEMPERATURE (°C)

in Section II-7B. The components of the endotherms are also shown in Figure III-3. At cholesterol concentrations greater than 17mol% the endotherms appeared to consist of single broad components.

i. Total Transition Enthalpies

For any given concentration of cholesterol the decrease in the maximum excess heat capacity and the prominence of the high-temperature asymmetry were greater in OSPC-cholesterol than in SOPC-cholesterol. The greater effect of cholesterol on the behavior of OSPC than on that of SOPC was reflected in the total enthalpies of the transitions as shown in Figure III-4. No transitions were detected with dispersions of OSPC containing 30mol% cholesterol. A broad transition was observed with SOPC containing 30mol% cholesterol but no transition could be detected with SOPC-cholesterol at $X_{chol} = 0.40$.

ii. Enthalpies Associated with the Components of the Endotherms

The enthalpies associated with the broad components increased on addition of cholesterol up to 17mol% cholesterol (Figure III-5A). Addition of cholesterol above 17mol% caused a decrease in the enthalpies of the broad endotherms for mixtures of cholesterol with both SOPC and OSPC. Figure III-5B shows the effects of cholesterol concentration on the enthalpies associated with the narrow com-

Figure III-A

Transition enthalpies of mixtures of cholesterol with SOPC (▲: containing 6mol% OSPC; ■: containing 11mol% OSPC) and with OSPC (△: containing 4mol% SOPC; □: containing <1mol% SOPC).

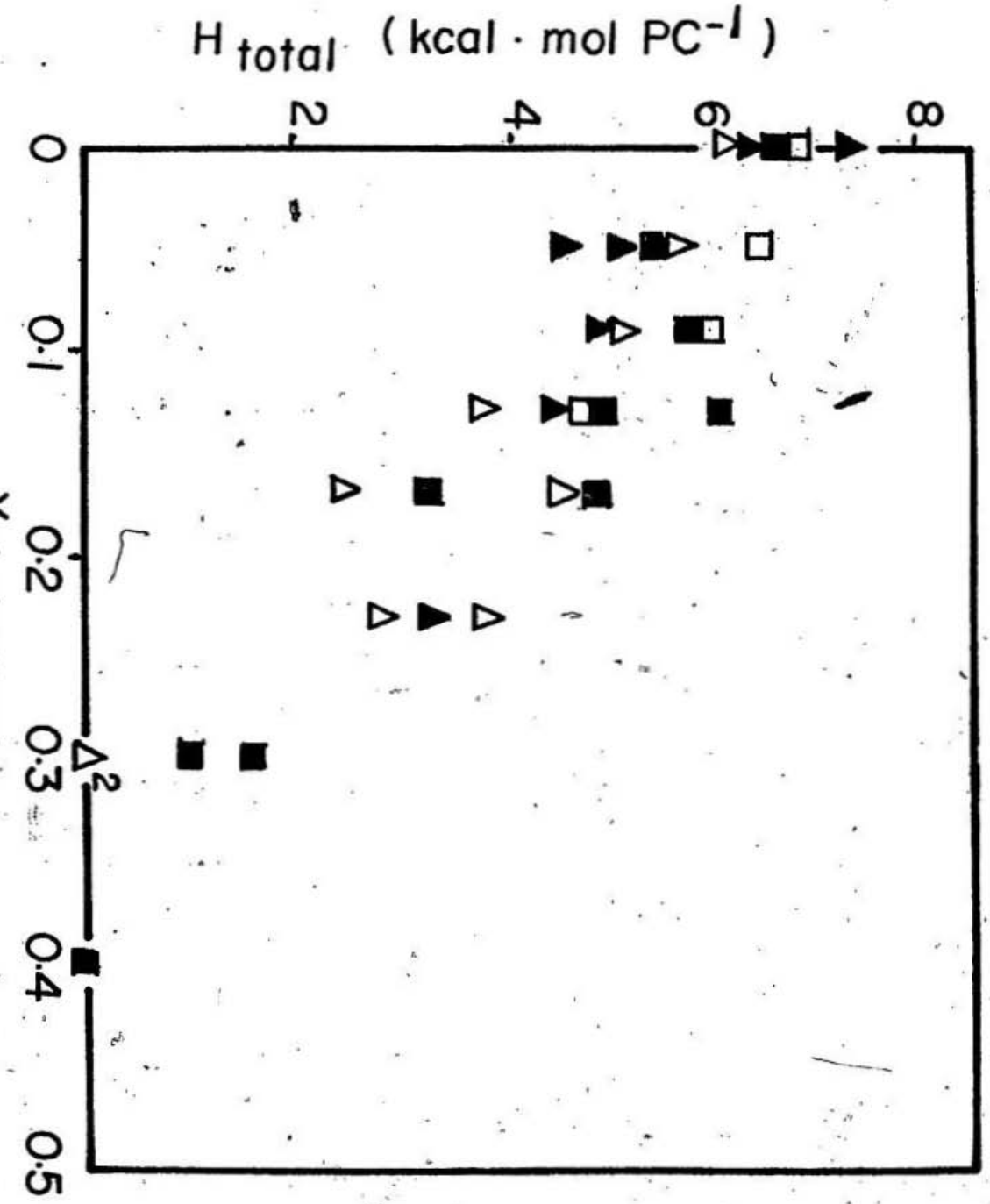
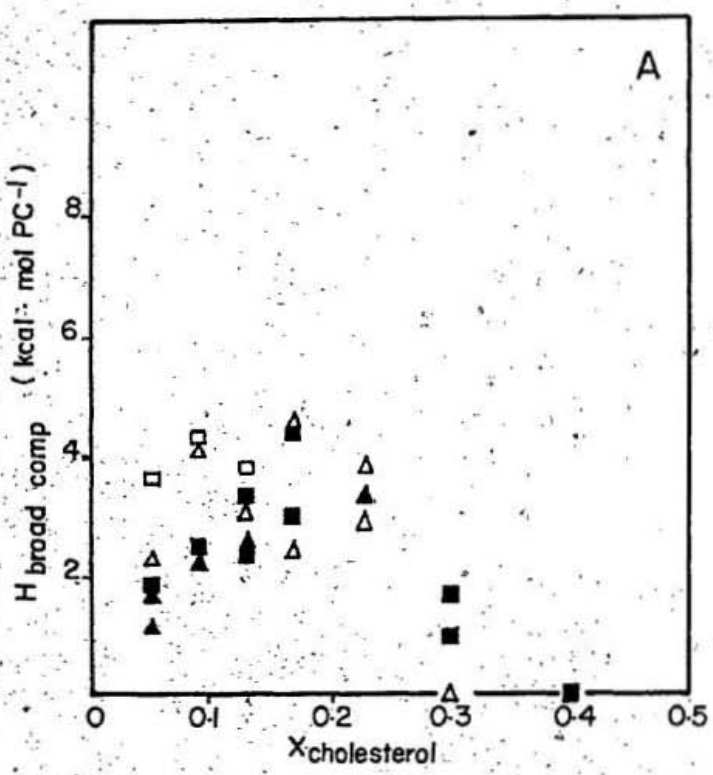
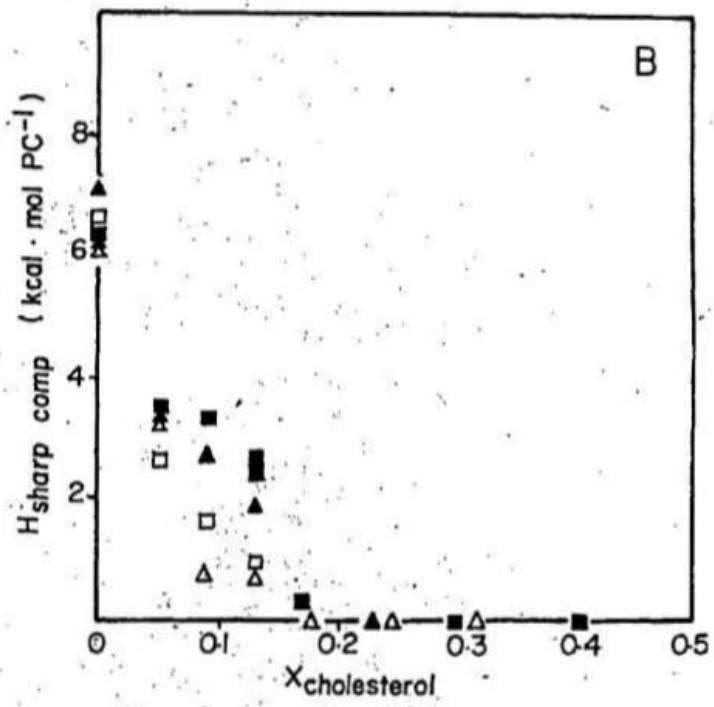

H total (kcal · mol PC⁻¹)
2
4
6
8
0
0·1
0·2
0·3
0·4
0·5
X cholesterol
Δ²

Figure III-5

The enthalpies associated with the broad (A) and narrow (B) components of the endotherms obtained with mixtures of cholesterol with SOPC (▲: 6% acyl migrated; ■: 11% acyl migrated) and with OSPC (△: 4% acyl migrated; □: <1% acyl migrated).


A
$H_{broad\ comp}$ (kcal · mol PC^{-1})
8
6
4
2
0
0·1
0·2
0·3
0·4
0·5
$X_{cholesterol}$


B
$H_{sharp\ comp}$ (kcal · mol PC^{-1})
8
6
4
2
0
0·1
0·2
0·3
0·4
0·5
$X_{cholesterol}$

ponents of these endotherms. The enthalpies of the narrow components of the endotherms decreased progressively on addition of cholesterol to OSPC and to SOPC but the narrow components of the endotherms were smaller in OSPC-cholesterol than in SOPC-cholesterol at any given cholesterol concentration from 5 to 17mol%. This difference in the effects of cholesterol on the narrow components is also illustrated in Figure III-6 where the relative contributions of the narrow components to the total endotherms are plotted against the cholesterol concentration. These are not restatements of the plots shown in Figure III-5B although the shapes are similar. For each cholesterol concentration, the narrow endotherms of OSPC-cholesterol contributed a small portion of the total enthalpy of the transitions than did the narrow components of SOPC-cholesterol.

iii. Temperatures and Widths of Components of the Endotherms

Figure III-7 shows the effects of cholesterol concentration on the temperatures of maximum excess heat capacity (T_{max}) and the widths at half maximum excess heat capacity ($\Delta T_{\frac{1}{2}}$) of the components of the endotherms obtained by deconvolution of the OSPC-cholesterol and SOPC-cholesterol endotherms. The values of T_{max} of the narrow components of the endotherms decreased progressively on addition of cholesterol to SOPC until the narrow components disappeared. Changes in the T_{max} of the narrow components of the endoth-

Figure III-6

The relative contribution of the narrow components of the endotherms to the total transition enthalpies of mixtures of cholesterol with SOPC (▲: 6% acyl migrated: ■ : 11% acyl migrated) and with OSPC (△: 4% acyl migrated; □ : <1% acyl migrated).

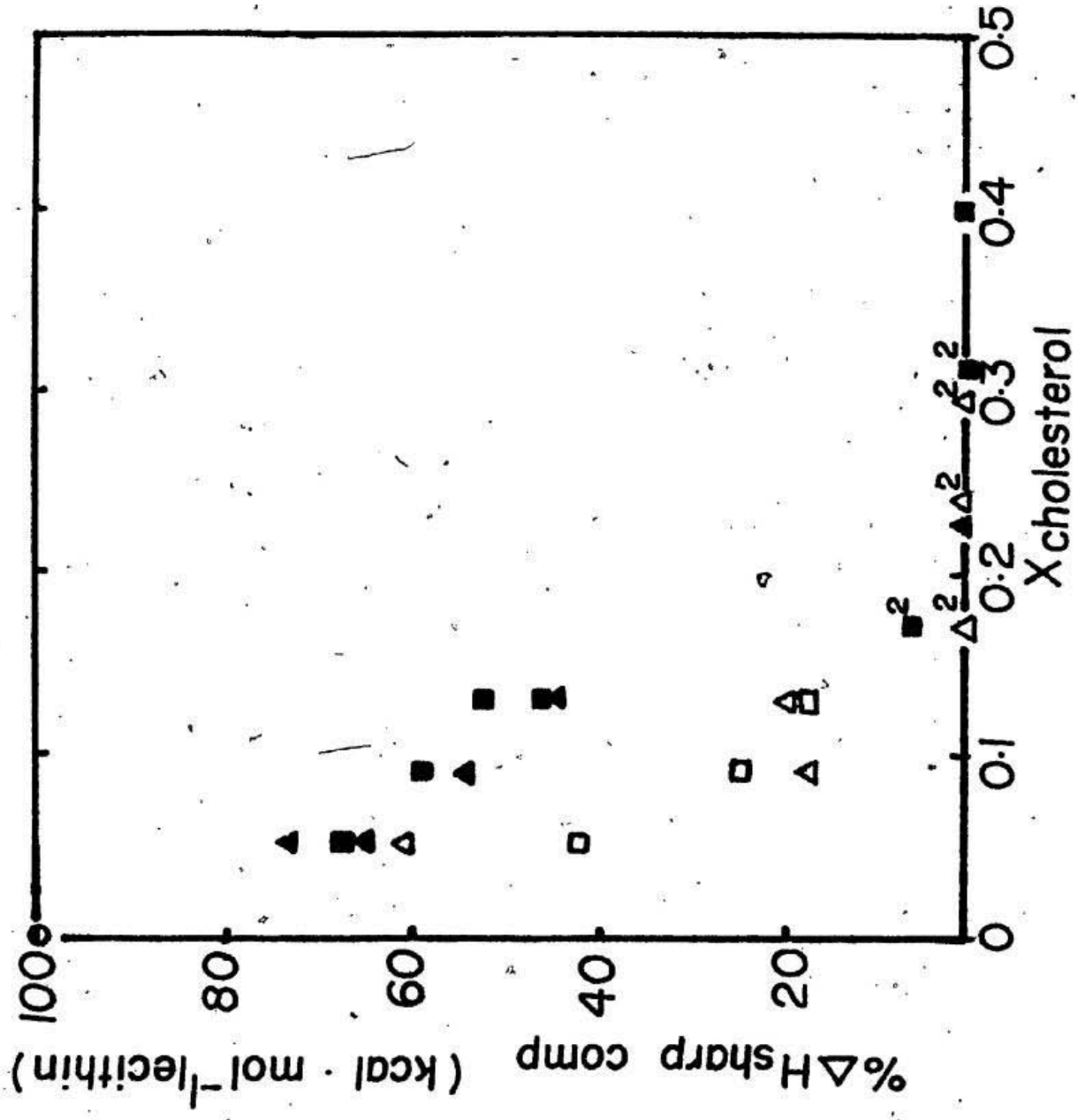
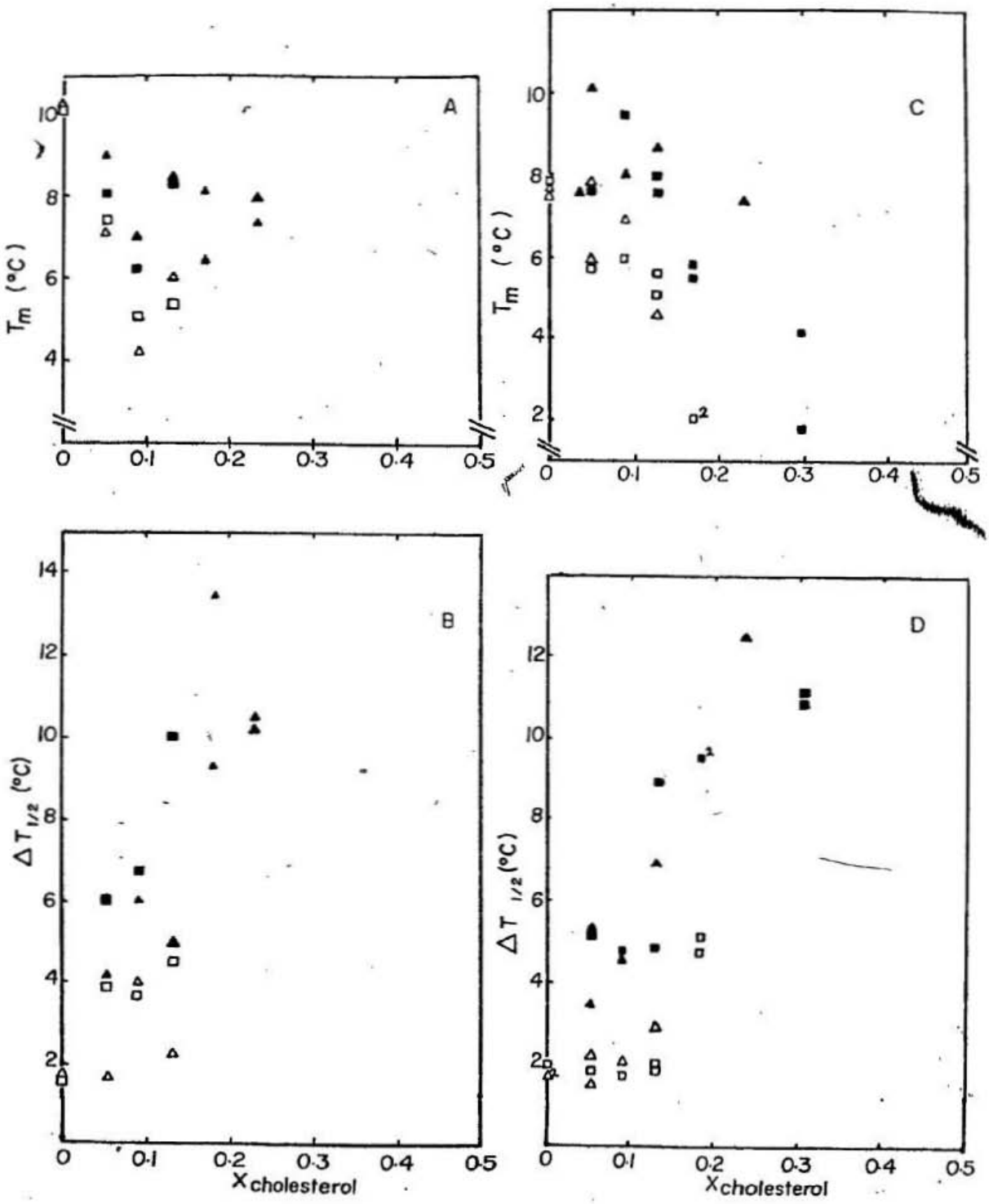

%ΔH sharp comp (kcal · mol⁻¹ lecithin)
100
80
60
40
20
0
0·1
0·2
0·3
0·4
0·5
X cholesterol

Figure III-7

The temperatures of maximum excess heat capacity (T_{max}) and the width at half maximum excess heat capacity ($\Delta T_{\frac{1}{2}}$) of the broad (closed symbols) and narrow (open symbols) components of the endotherms of mixtures of cholesterol with OSPC (A, B; ▲, △ : 4% acyl migration; ■, □ : <1% acyl migration) and with SOPC (C, D; ▲, △ : 6% acyl migration; ■, □ : 11% acyl migration).


A
B
C
D
T_m (°C)
$\Delta T_{1/2}$ (°C)
$X_{cholesterol}$
0
0·1
0·2
0·3
0·4
0·5

-erms of OSPC-cholesterol were less pronounced. The T_{max} values of the broad components of endotherms of SOPC-cholesterol mixtures decreased with increasing cholesterol concentration while the T_{max} of the broad endotherms from OSPC-cholesterol mixtures were relatively insensitive to changes in the cholesterol concentration.

The $\Delta T_{\frac{1}{2}}$ values of the narrow components of the endotherms in both OSPC-cholesterol mixtures and SOPC-cholesterol mixtures were nearly independent of the concentration of cholesterol, but the variability in the $\Delta T_{\frac{1}{2}}$ values was fairly large. The $\Delta T_{\frac{1}{2}}$ values for the broad components of the endotherms in both sets of mixtures increased from a range of 4-5K at 5mol% cholesterol, to a range of 10-12K at 23mol%.

B. Effects of Cholesterol on OAPC and AOPC

Typical heating endotherms (normalized per mole of lipid phosphorus) obtained from dispersions of mixtures of cholesterol with OAPC and with AOPC are shown in Figure III-8. In these mixtures there was also a progressive decrease in the maximum excess heat capacity as the cholesterol concentration increased. For any given concentration of cholesterol, the maxima of the excess heat capacity of AOPC-cholesterol endotherms were lower than those of OAPC-cholesterol endotherms.

In AOPC containing 5mol% cholesterol the heating

endotherms had a pronounced high-temperature shoulder. This shoulder persisted up to 17mol% cholesterol and at greater than 17mol% only a symmetric broad endotherm was observed. No high-temperature asymmetry was observed in the endotherms of OAPC-cholesterol mixtures containing less than 13mol% cholesterol. At 13mol% and 17mol% cholesterol in OAPC there appeared to be some evidence for the presence of two components in the heating endotherms.

These endotherms were subjected to the same deconvolution procedure used for the endotherms of the mixtures of cholesterol with OSPC and SOPC, and the component curves are shown in Figure III-8. The OAPC-cholesterol endotherms obtained at X_{chol} = 0.05 and 0.10 were resolved into two components even though it is possible that these endotherms may contain only one component.

i. Total Enthalpies of the Transitions

The effects of cholesterol concentration on the total enthalpies of the transitions in OAPC-cholesterol and AOPC-cholesterol mixtures are shown in Figure III-9. For concentrations of cholesterol between 5 and 23mol% the total enthalpies of the transitions of AOPC-cholesterol mixtures were lower than those of OAPC-cholesterol mixtures at any given cholesterol concentrations. No transition endotherms could be resolved from the baseline in AOPC-cholesterol mixtures containing 40mol% cholesterol. A transition was detected in OAPC plus 40mol% cholesterol but was not

Figure III-8

Normalized dsc endotherms obtained with aqueous dispersions of mixtures of cholesterol with OAPC and with AOPC, and the components of the endotherms. (+) points from the normalized dsc endotherms; (——) composite endotherms, (.....) narrow components of the endotherms, and (– – –) broad components of the endotherms obtained using the computer analysis described in Section-7F.

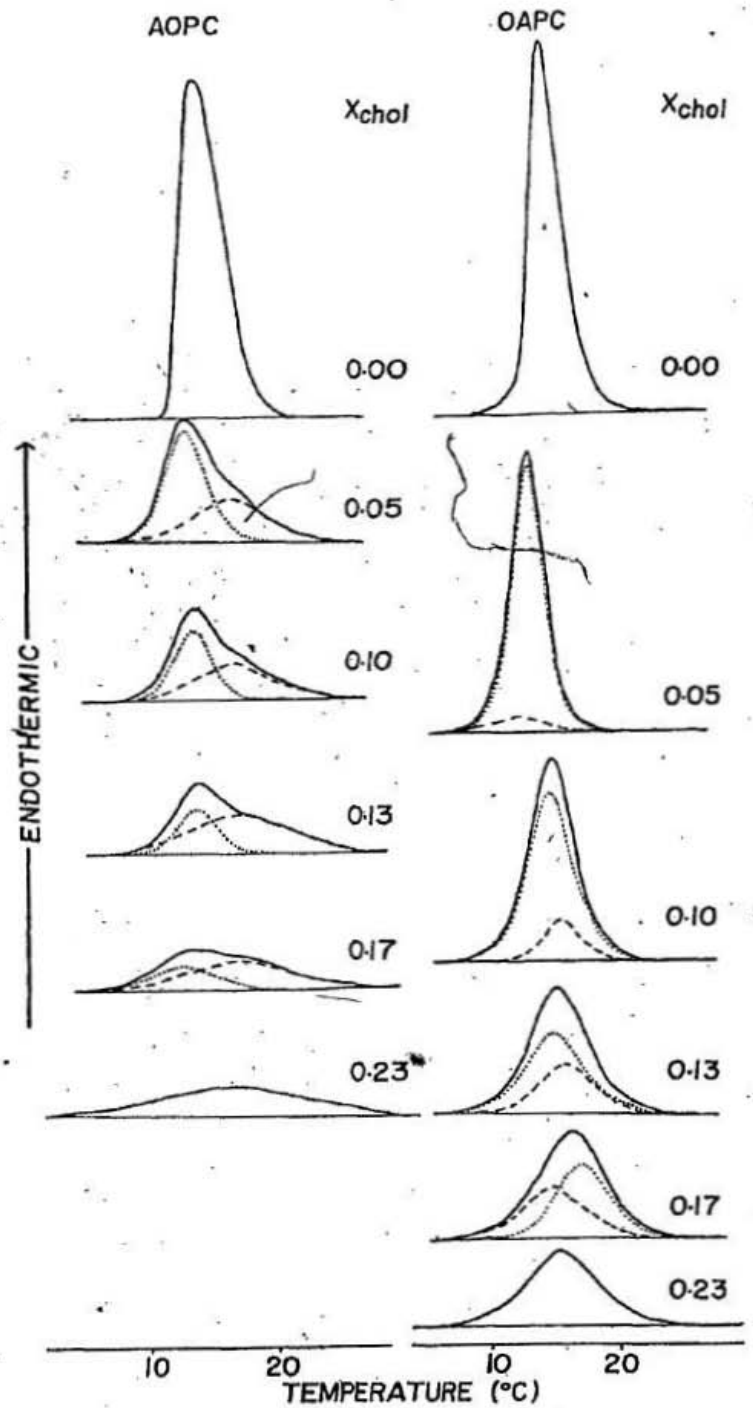
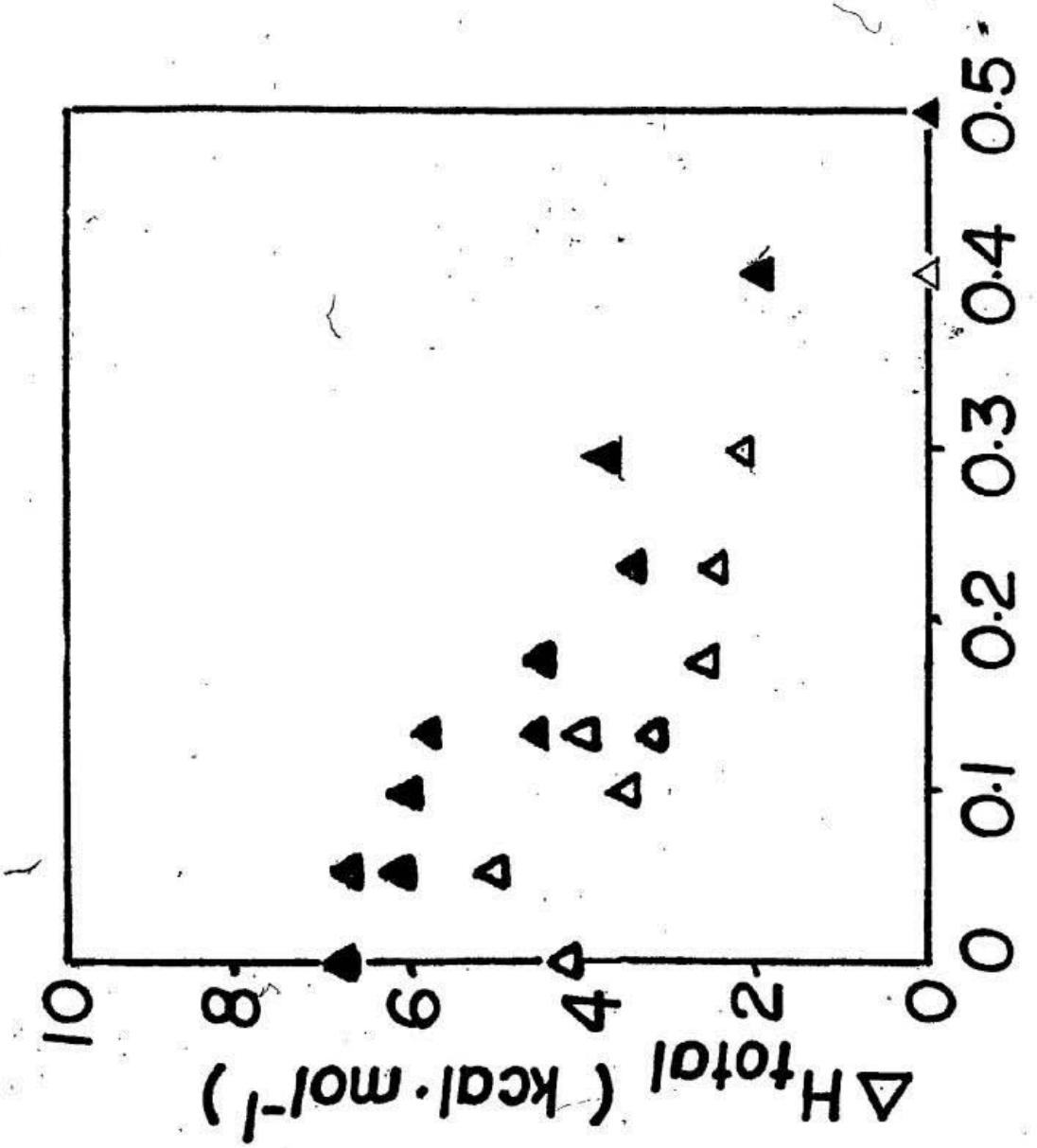

AOPC
OAPC
Xchol
Xchol
0·00
0·00
0·05
0·10
0·05
0·13
0·10
0·17
0·23
0·13
0·17
0·23
ENDOTHERMIC
10
20
10
20
TEMPERATURE (°C)

Figure III-9

Transition enthalpies of mixtures of cholesterol with AOPC (△) and with OAPC (▲).


ΔH_total (kcal·mol⁻¹)
10
8
6
4
2
0
0
0.1
0.2
0.3
0.4
0.5
X_cholesterol

detected in OAPC plus 50mol% cholesterol.

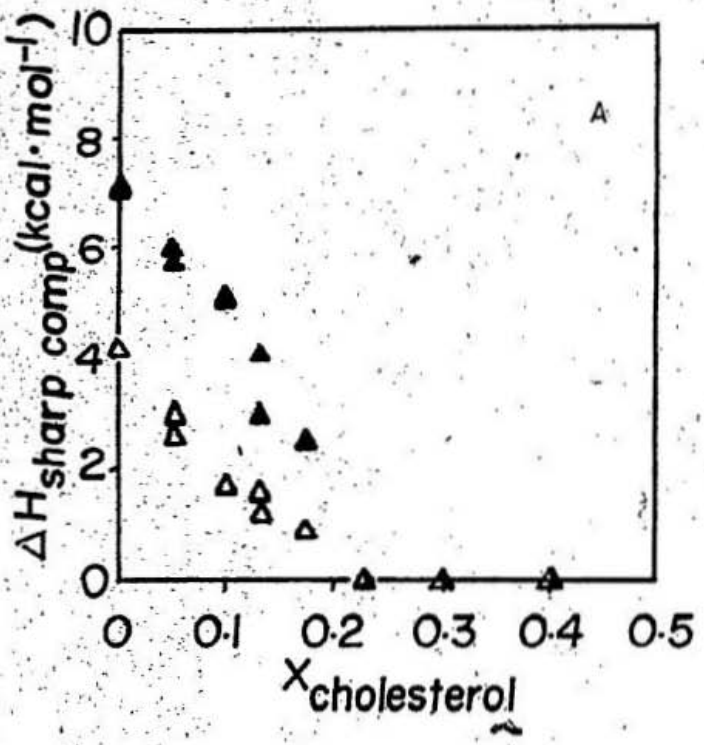
ii. Enthalpies of the Components of the Endotherms

The enthalpies associated with the narrow components of the endotherms obtained by deconvolution of the OAPC-cholesterol and AOPC-cholesterol endotherms for X_{chol} = 0.05 to 0.17 decreased as the cholesterol concentration increased (Figure 10A). At $X_{chol} \geq 0.23$ the endotherms observed in both mixtures appeared to be broad symmetric endotherms (Figure III-8). The enthalpies attributable to the broad components of the endotherms in OAPC-cholesterol increased as the cholesterol concentration increased from 5 to 23mol% (Figure III-10B). In AOPC-cholesterol, the enthalpies of the broad component appeared to remain unchanged by addition of cholesterol above X_{chol} = 0.05 at least up to X_{chol} = 0.23.

The enthalpies associated with the narrow component of the endotherms of AOPC-cholesterol mixtures were smaller than those of the corresponding OAPC-cholesterol mixtures in the range of 5 to 17mol% cholesterol (Figure III-10A). At any given concentration of cholesterol between 5 and 13mol% the enthalpies of the broad components of the endotherms of AOPC-cholesterol mixtures were greater than those of OAPC-cholesterol mixtures. At any given concentration of cholesterol the enthalpies of the narrow components of the endotherms also represented smaller fractions of the total enthalpies of the transitions for AOPC-cholesterol than for

Figure III-10

The enthalpies associated with the narrow (A) and broad (B) components of the endotherms obtained with mixtures of cholesterol with OAPC (▲, ▲) and with AOPC (△, △).


A
$\Delta H_{sharp\ comp}$ (kcal·mol^{-1})
10
8
6
4
2
0
0
0·1
0·2
0·3
0·4
0·5
$X_{cholesterol}$

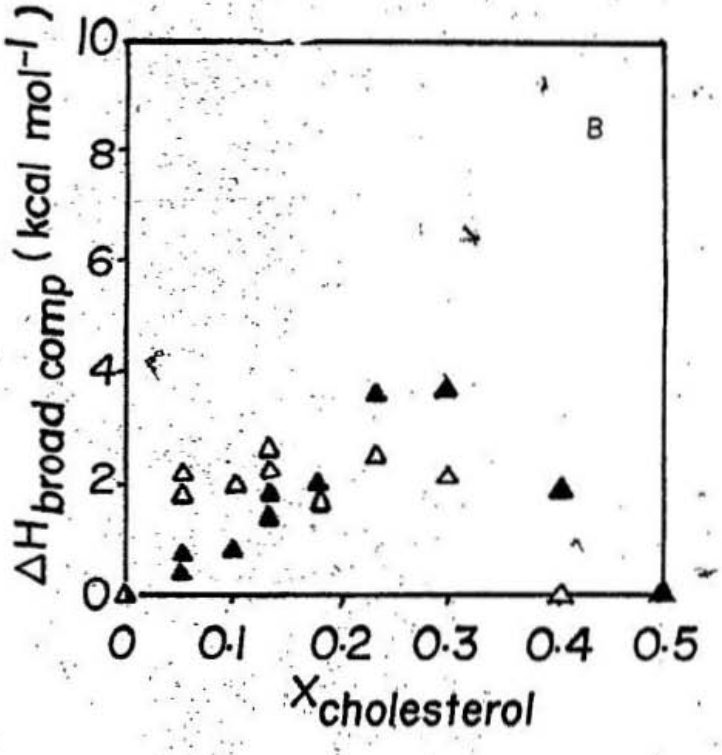
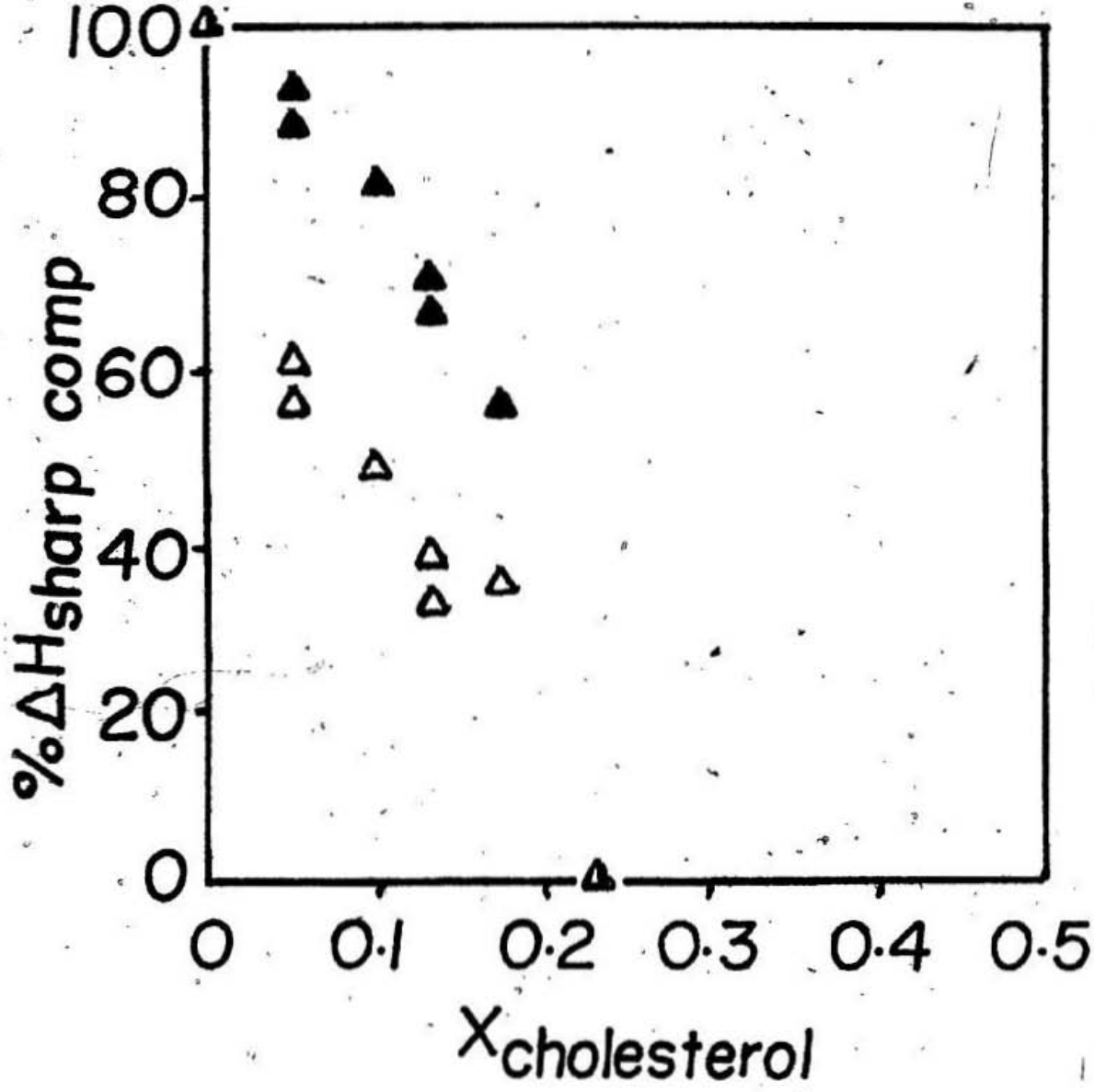

B
$\Delta H_{broad\ comp}$ (kcal mol^{-1})
10
8
6
4
2
0
0
0·1
0·2
0·3
0·4
0·5
$X_{cholesterol}$

Figure III-11

The relative contributions of the narrow components of the endotherms to the total transition enthalpies of mixtures of cholesterol with OAPC (▲) and with AOPC (△).


100
80
60
40
20
0
%ΔHsharp comp
0
0·1
0·2
0·3
0·4
0·5
Xcholesterol

OAPC-cholesterol (Figure III-11).

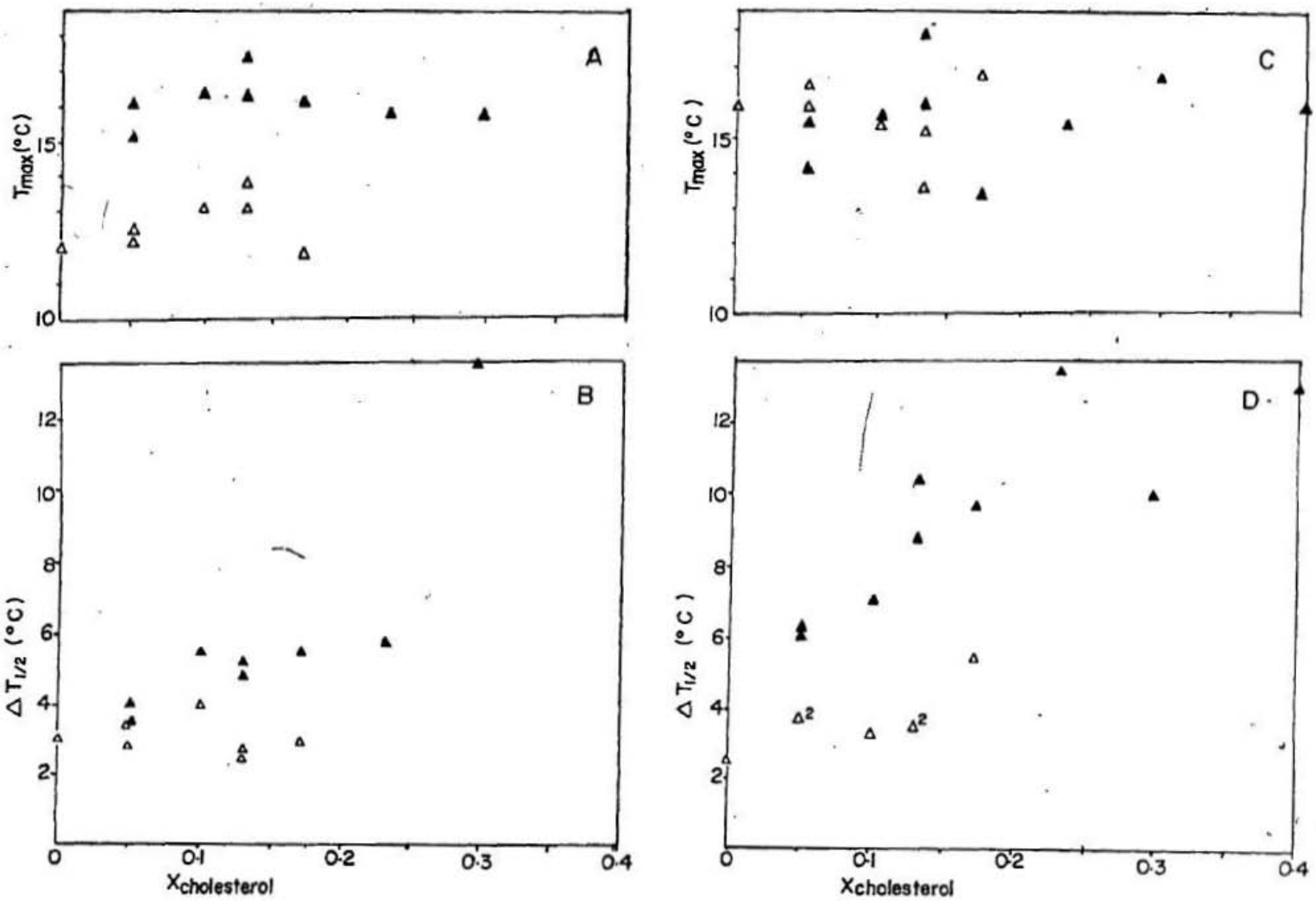
iii. Temperatures and Widths of the Components of the Endotherms

The effects of cholesterol concentration on the T_{max} of the narrow and broad components of the endotherms obtained from the mixtures of OAPC-cholesterol and AOPC-cholesterol are shown in Figures III-12A and III-12C. The values of T_{max} of the narrow components obtained with AOPC-cholesterol were independent of the cholesterol concentration and were the same as the T_{max} of pure AOPC. The values of T_{max} of the broad components were 3 to 4 degrees higher than those of the narrow components in the mixtures of AOPC and cholesterol and the values of T_{max} of the broad components did not change with increasing cholesterol concentration. In the mixtures of OAPC with cholesterol the T_{max} values of both the narrow and broad components of the endotherms showed more variability than those of the other mixtures but the T_{max} values of both components appeared to be independent of the cholesterol concentration.

The values of $\Delta T_{\frac{1}{2}}$ of the components of the endotherms are plotted against the cholesterol concentration for the mixtures of cholesterol with OAPC and AOPC in Figures III-12B and III-12D. In the mixtures with AOPC, the $\Delta T_{\frac{1}{2}}$ of the narrow components increased slightly as the cholesterol concentration increased. The $\Delta T_{\frac{1}{2}}$ values of the broad components increased progressively as more cholesterol was

Figure III-12

The temperatures of maximum excess heat capacity (T_{max}) and the half height widths ($\Delta T_{\frac{1}{2}}$) of the broad (▲) and narrow (△) components of the endotherms of mixtures of cholesterol with AOPC (A, B) and with OAPC (C, D). Superscript (2) on symbols indicates two coincident points.


A
B
C
D
T_{max} (°C)
$\Delta T_{1/2}$ (°C)
$X_{cholesterol}$
10
15
2
4
6
8
10
12
0
0·1
0·2
0·3
0·4

added. In OAPC-cholesterol, the $\Delta T_{\frac{1}{2}}$ values of both the narrow and broad components were independent of the cholesterol concentration.

C. Effect of Cholesterol on DOPC, DSPC and DAPC.

Figure III-13 shows typical normalized endotherms obtained for mixtures of cholesterol with the single-acid lecithins DOPC, DSPC and DAPC. Addition of cholesterol to each of these lecithins caused a decrease in the maximum excess heat capacities of the heating endotherms. The endotherms of the DOPC-cholesterol mixtures had a low-temperature asymmetry which became more pronounced with increasing concentrations of cholesterol. The endotherms of the DSPC-cholesterol mixtures were nearly symmetrical but they broadened with increases in the concentration of cholesterol. The endotherms obtained for DAPC-cholesterol were like those obtained for DOPC-cholesterol in that a low-temperature asymmetry was observed which increased with increasing cholesterol concentrations. The endotherms obtained for these mixtures were resolved into two components and the components of the endotherms are shown in Figure II-13.

1. Effects of Cholesterol Concentration on the Total Enthalpies of the Transitions.

The effects of the concentration of cholesterol on the enthalpies of the transitions for mixtures of cholesterol

Figure III-13

Normalized dsc endotherms obtained on heating aqueous dispersions of mixtures of cholesterol with DOPC (A), with DSPC (B) and with DAPC (C), and the components of the endotherms. (+) points from the normalized dsc endotherms; (——) composite endotherms, (·····) narrow components of the endotherms, and (– – –) broad components of the endotherms obtained using the computer analysis described in Section II-7E.

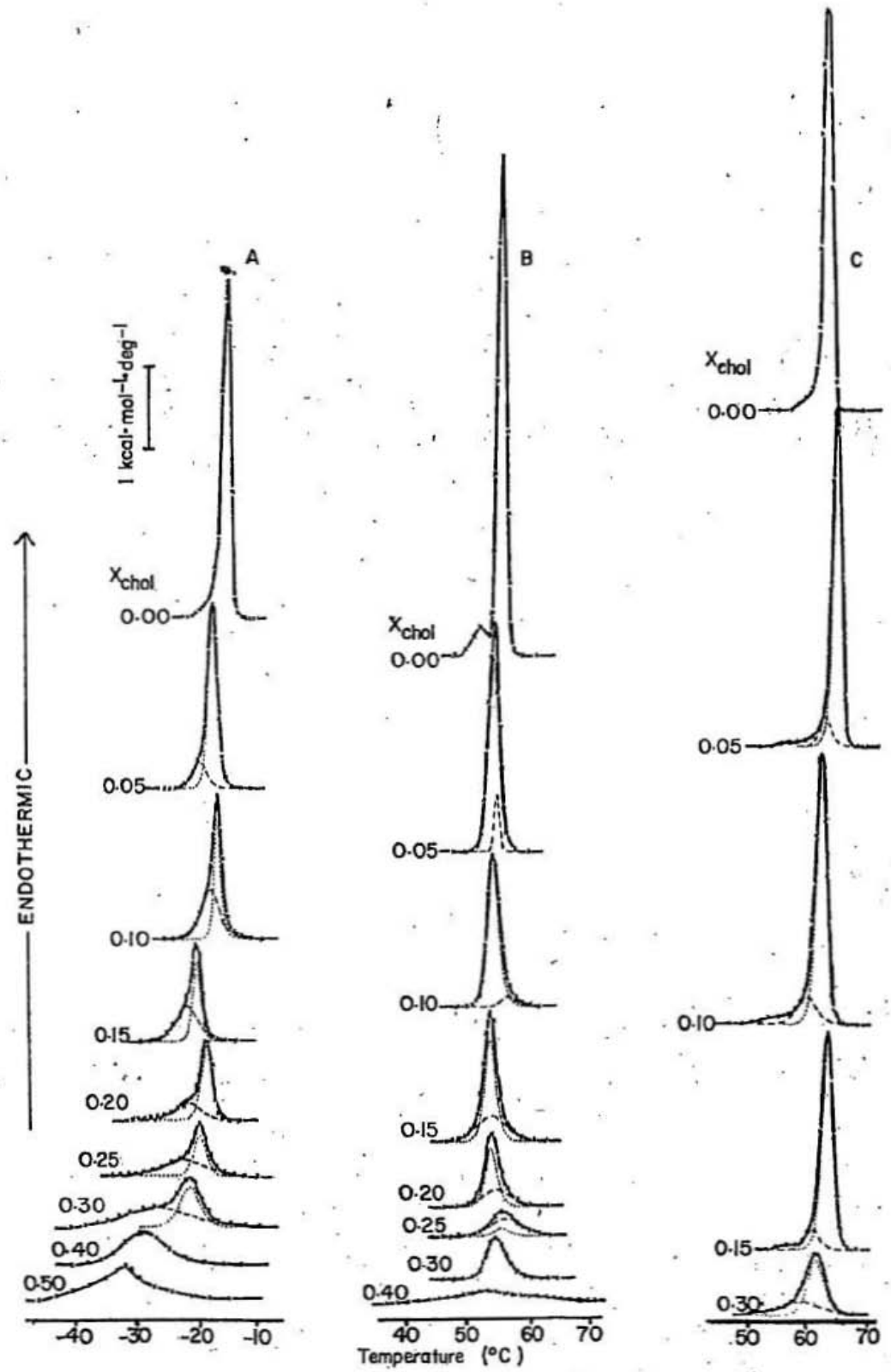
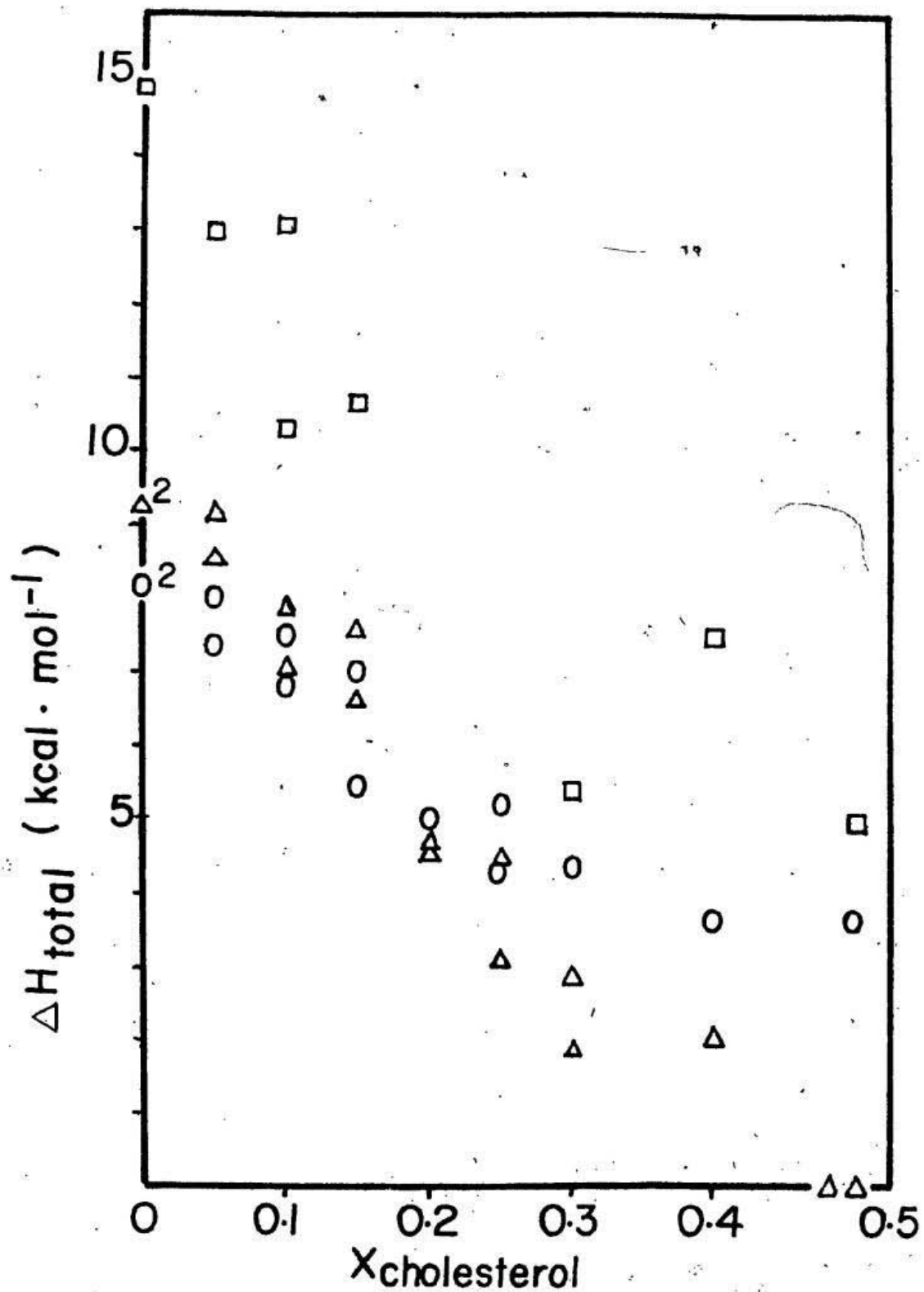

A
B
C
1 kcal·mol⁻¹·deg⁻¹
X_chol
0·00
0·05
0·10
0·15
0·20
0·25
0·30
0·40
0·50
ENDOTHERMIC
-40 -30 -20 -10
40 50 60 70
Temperature (°C)
50 60 70

Figure III-14

Transition enthalpies of mixtures of cholesterol with DOPC (○), with DSPC (△), and with DAPC (□).


15
10
5
0
0·1
0·2
0·3
0·4
0·5
ΔH_{total} (kcal · mol^{-1})
$X_{cholesterol}$

with DOPC, DSPC and DAPC are shown in Figure III-14. Addition of cholesterol caused a progressive decrease in the total enthalpies of the transitions up to 30mol% cholesterol in DOPC and DAPC and up to 48mol% cholesterol in DSPC. In the latter case, the transition could not be resolved from the baseline at X_{chol} = 0.48. In DOPC-cholesterol and DAPC-cholesterol addition of cholesterol above 30mol% did not result in any further reduction in the enthalpies of the transitions.

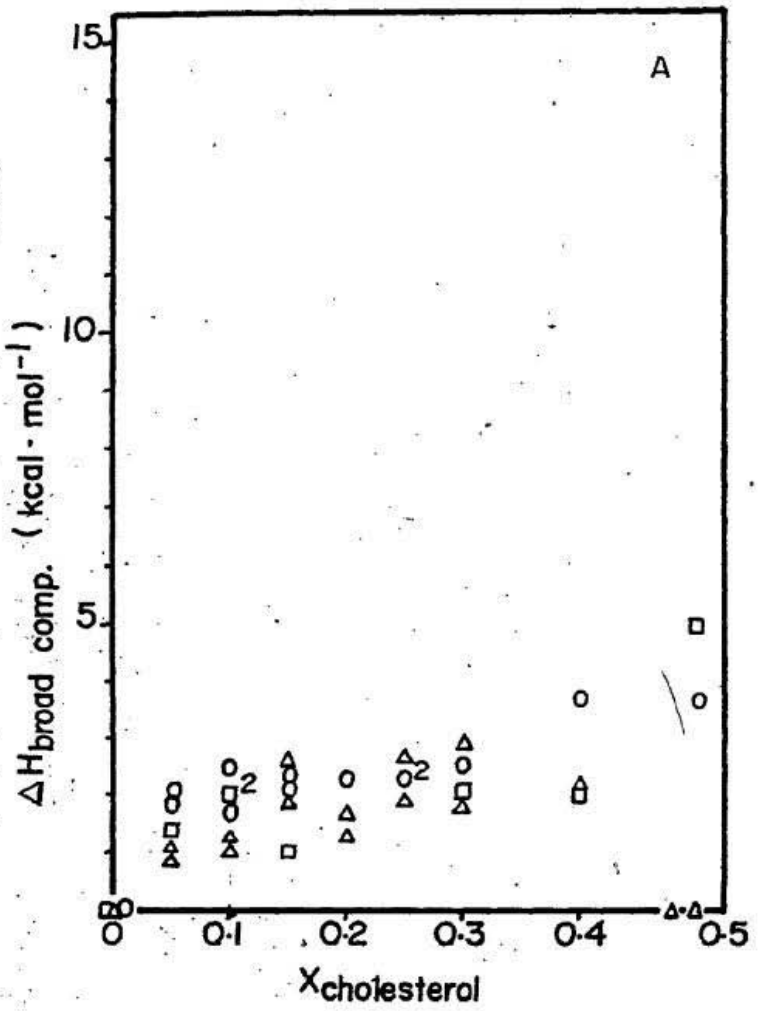
ii. Effects of Cholesterol Concentration on the Enthalpies of the Components of the Endotherms

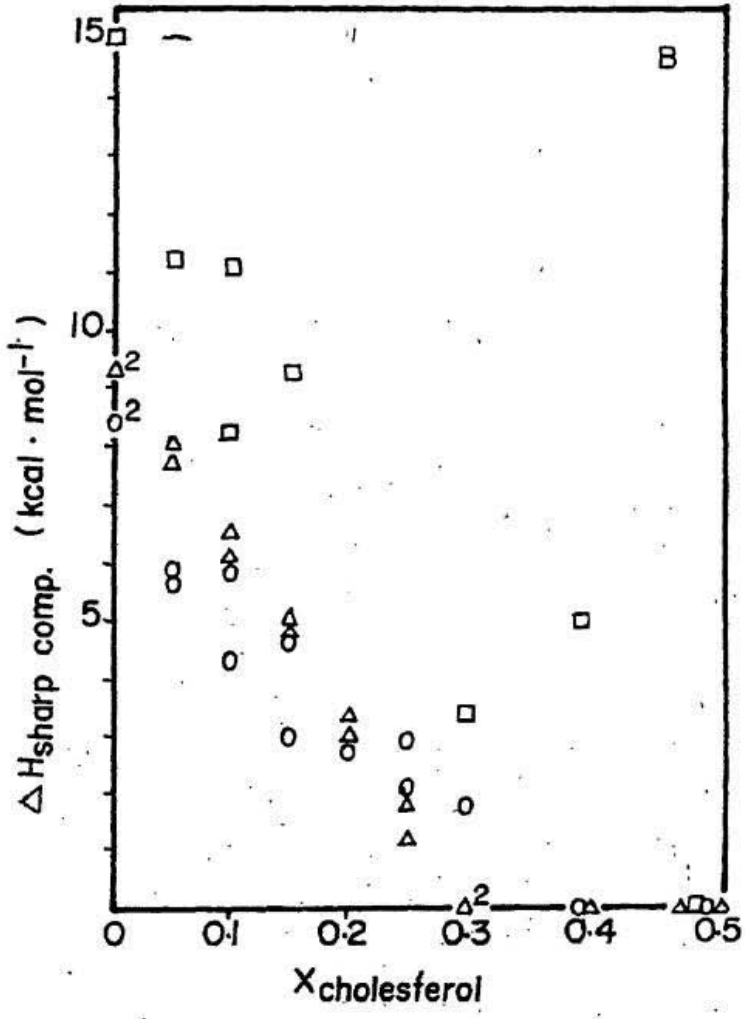
The effects of the cholesterol concentration on the enthalpies of the broad components of these endotherms are shown in Figure III-15A. There was little, if any, difference between the enthalpies of the broad components for the three lecithin-cholesterol mixtures up to 30mol% cholesterol. Beyond 40mol% cholesterol in DSPC, the enthalpies of the broad components were reduced to zero but the enthalpies of the broad components for DAPC-cholesterol increased as the cholesterol concentration increased from 40mol% to 50mol%. The enthalpies of the broad components for DOPC-cholesterol were unchanged by addition of more cholesterol.

The enthalpies associated with the narrow components of the endotherms obtained for these mixtures decreased progressively as the cholesterol concentration increased

Figure III-15

The enthalpies associated with the broad (A) and narrow (B) components of the endotherms obtained with mixtures of cholesterol with DOPC (○), with DSPC (△), and with DAPC (□).


A
15
10
5
0
0·1
0·2
0·3
0·4
0·5
ΔH broad comp. (kcal · mol⁻¹)
X cholesterol


B
15
10
5
0
0·1
0·2
0·3
0·4
0·5
ΔH sharp comp. (kcal · mol⁻¹)
X cholesferol

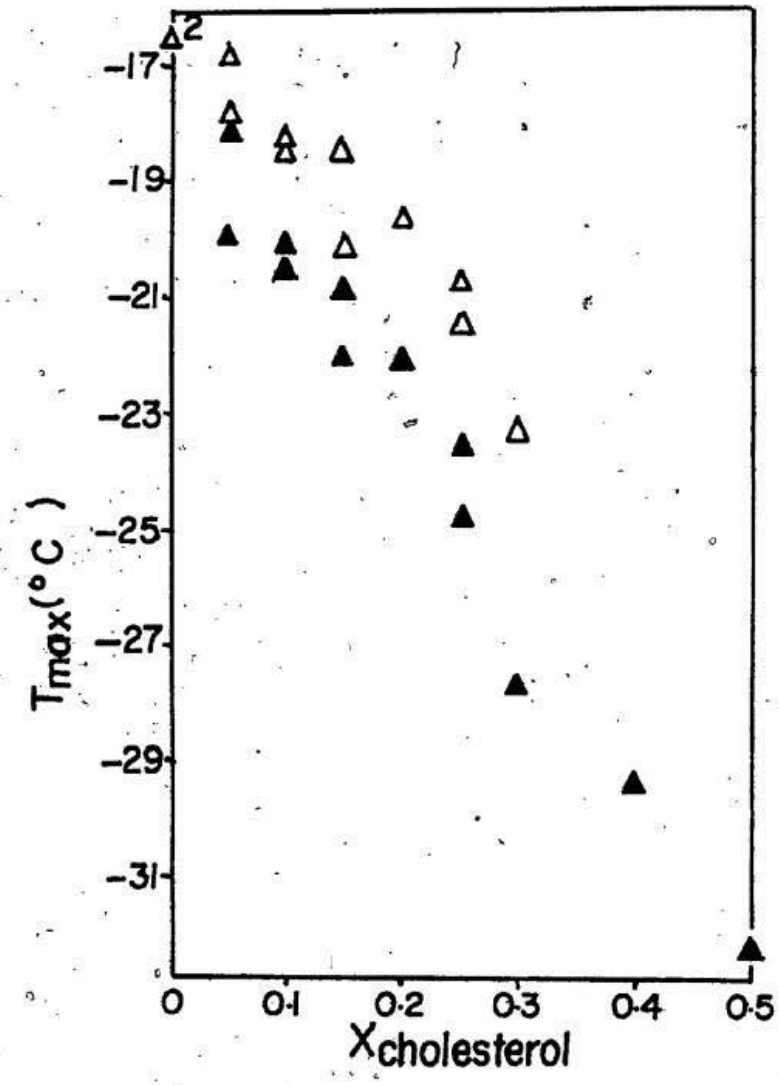
(Figure III-15B). The decreases in the enthalpies of the narrow components were linear for all three mixtures. The cholesterol concentrations at which the narrow components of the endotherms disappeared were 30mol% cholesterol in DSPC, 40mol% in DOPC and 50mol% in DAPC. The behavior of these single-acid lecithin-cholesterol mixtures was different from the behavior of mixtures of cholesterol with DMPC and DPPC (Estep et al., 1978; Mabrey et al., 1978; Melchoir et al., 1980).

iii. Effects of Cholesterol Concentration on Temperatures and Widths of Components of the Endotherms.

The effects of cholesterol concentration on the T_{max} values and $\Delta T_{\frac{1}{2}}$ values of the components of the endotherms of DOPC-cholesterol are shown in Figure III-16. The addition of cholesterol caused a dramatic decrease in the T_{max} values of both the narrow and broad components. Again, it was noteworthy that the T_{max} of the broad components were below those of the narrow components. The $\Delta T_{\frac{1}{2}}$ values of the narrow components were independent of the cholesterol concentration in the DOPC-cholesterol mixtures but the $\Delta T_{\frac{1}{2}}$ of the broad components showed a progressive increase as more cholesterol was added. In mixtures of cholesterol and DSPC, the values of T_{max} for both the narrow and the broad components remained unchanged as the cholesterol concentration increased (Figure III-17). The $\Delta T_{\frac{1}{2}}$ values of the narrow components of the endotherms for DSPC-cholesterol did not

Figure III-16

The temperatures of maximum excess heat capacity (T_{max}) and the width at half maximum excess heat capacity ($\Delta T_{\frac{1}{2}}$) of the broad (▲) and narrow (△) components of the endotherms of mixtures of cholesterol with DOPC.


-17
-19
-21
-23
-25
-27
-29
-31
T_{max}(°C)
0
0·1
0·2
0·3
0·4
0·5
Xcholesterol

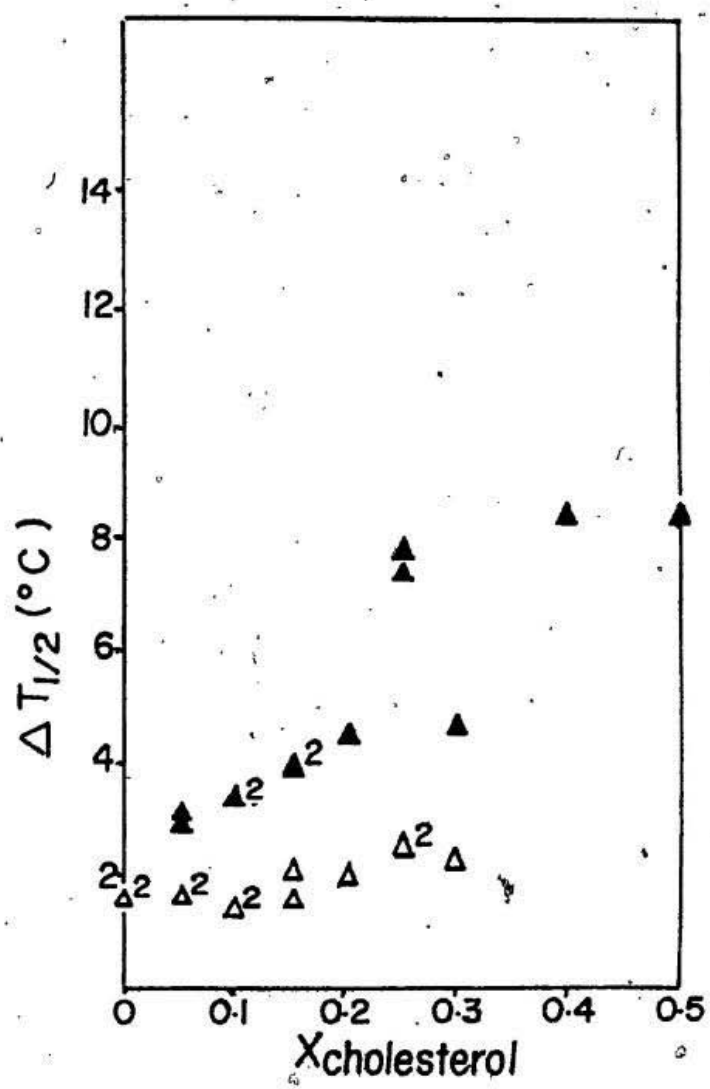

14
12
10
8
6
4
2
$\Delta T_{1/2}$ (°C)
0
0·1
0·2
0·3
0·4
0·5
Xcholesterol

Figure III-17

The temperatures of maximum excess heat capacity (T_{max}) and the widths at half maximum excess heat capacity ($\Delta T_{\frac{1}{2}}$) of the broad (▲) and narrow (△) components of the endotherms of mixtures of cholesterol with DSPC.

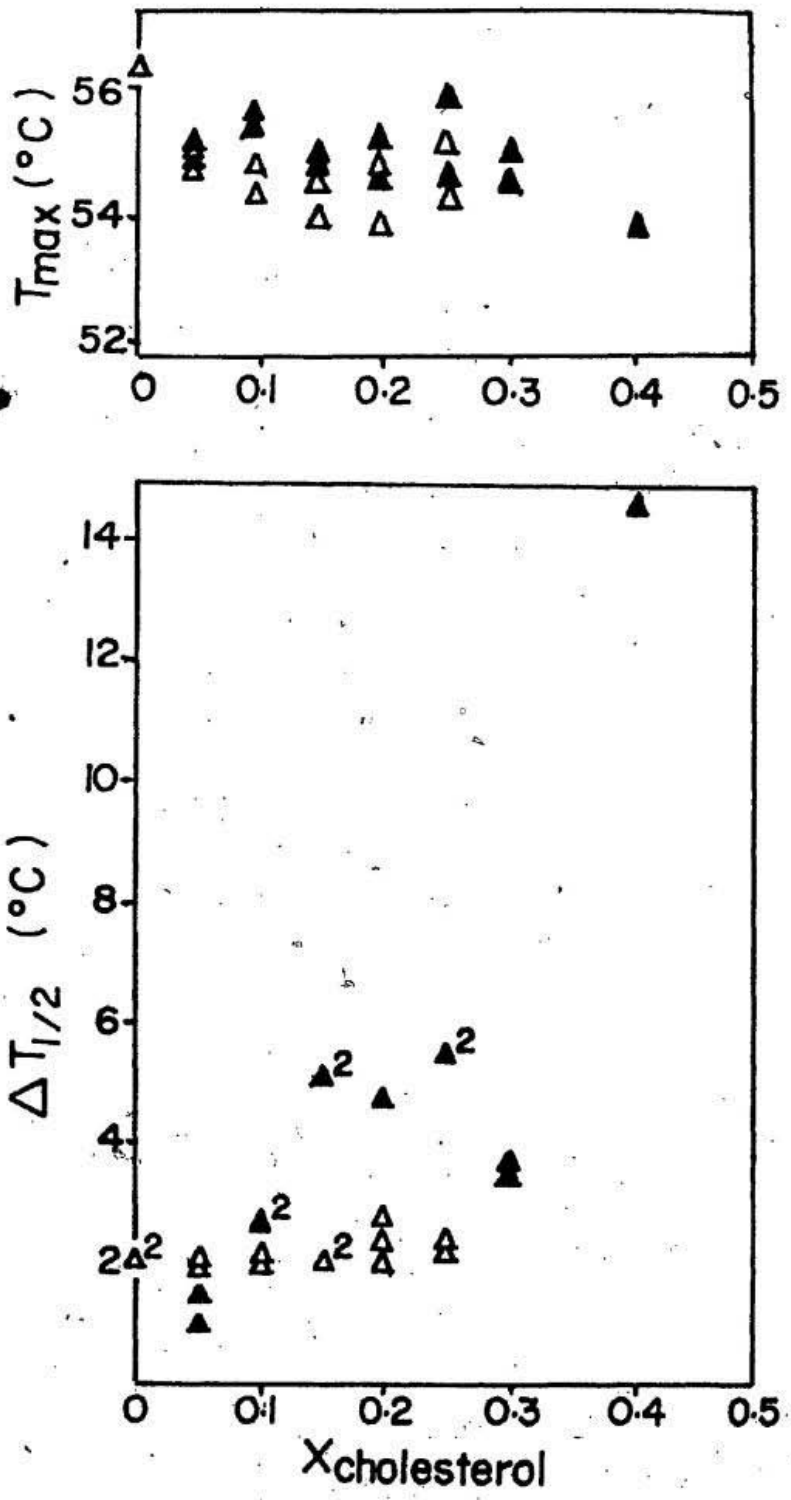
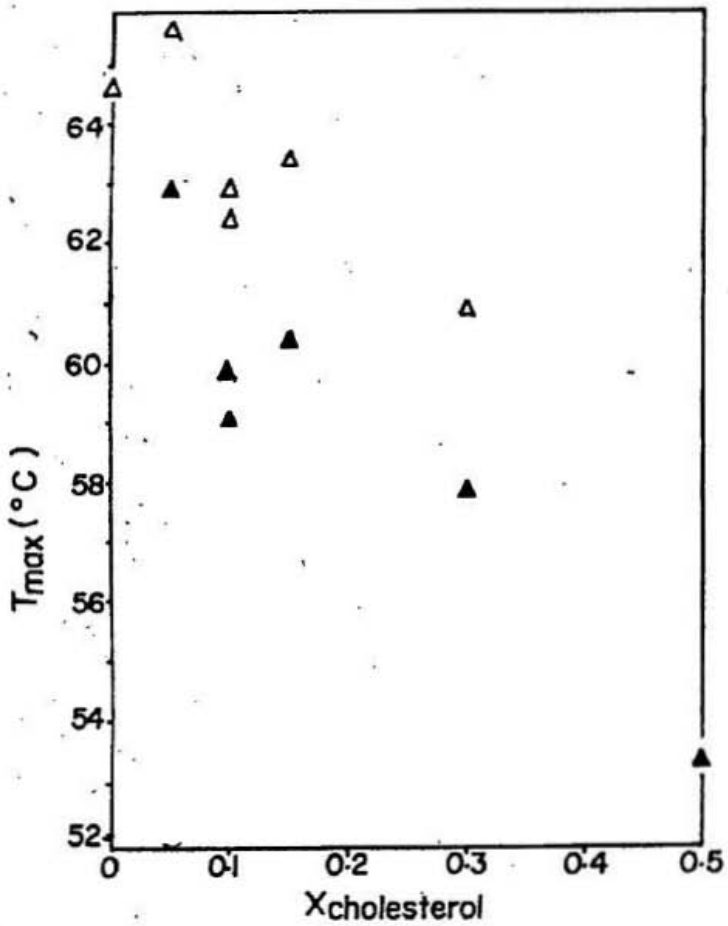
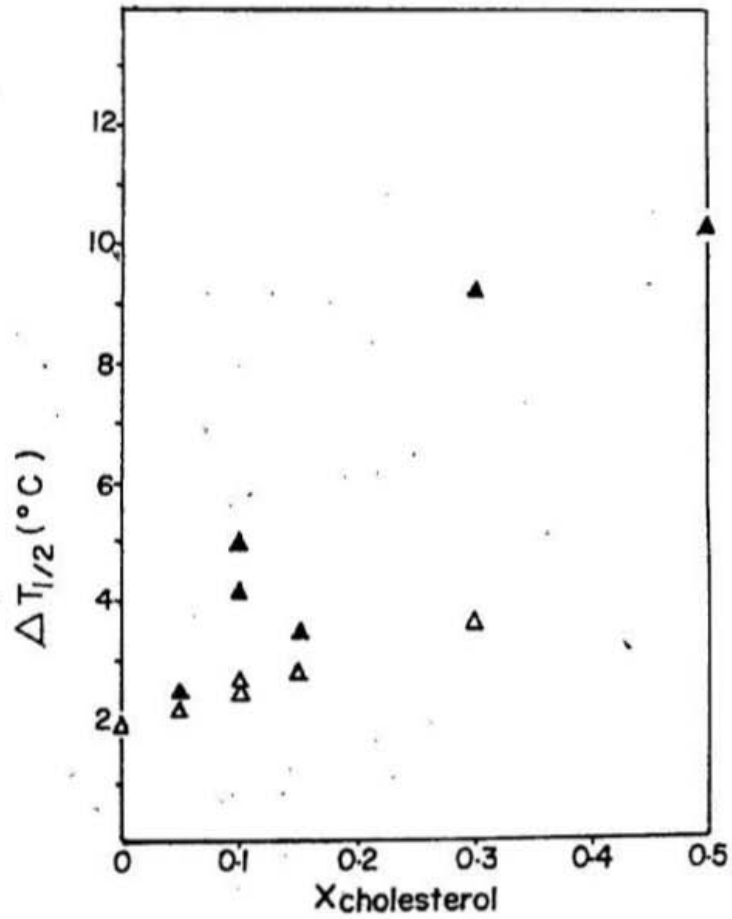

T_{max} (°C)
56
54
52
0
0·1
0·2
0·3
0·4
0·5
$\Delta T_{1/2}$ (°C)
14
12
10
8
6
4
2
$X_{cholesterol}$

Figure III-18

The temperatures of maximum excess heat capacity (T_{max}) and the widths at half maximum excess heat capacity ($\Delta T_{\frac{1}{2}}$) of the broad (▲) and narrow (△) components of the endotherms of mixtures of cholesterol with DAPC.


T_{max} (°C)
64
62
60
58
56
54
52
0
0·1
0·2
0·3
0·4
0·5
Xcholesterol


$\Delta T_{1/2}$ (°C)
12
10
8
6
4
2
0
0·1
0·2
0·3
0·4
0·5
Xcholesterol

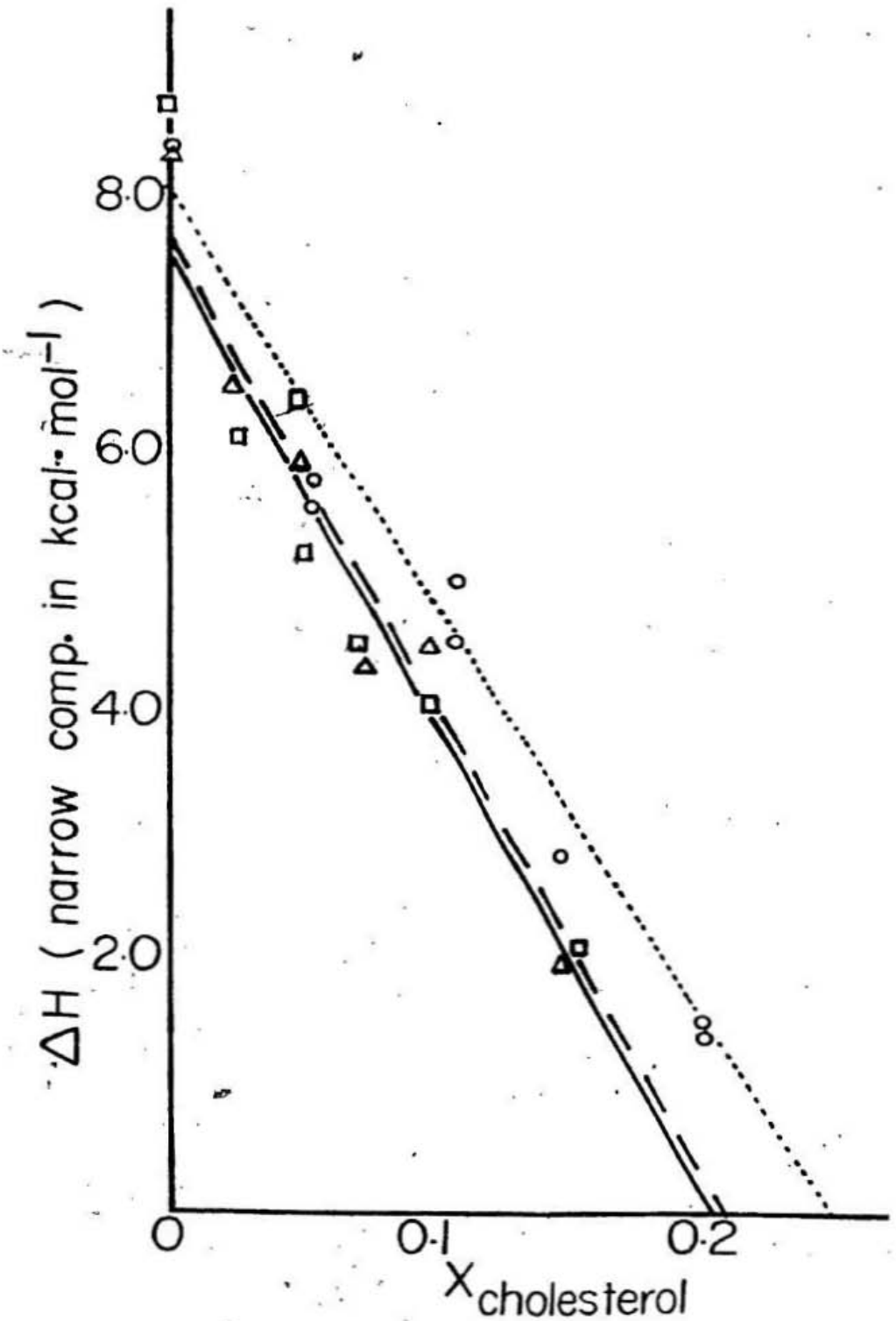
change over the range of cholesterol concentrations but the $\Delta T_{\frac{1}{2}}$ of the broad component increased with increasing concentrations of cholesterol (Figure III-17). Figure III-18 shows the effects of the cholesterol concentration on the values of T_{max} and $\Delta T_{\frac{1}{2}}$ of the narrow and broad components of the endotherms obtained for DAPC-cholesterol mixtures. The T_{max} values of both the narrow and broad components decreased with increasing cholesterol concentration, but the effect was less than was seen with the DOPC-cholesterol mixtures. The effects of cholesterol on the $\Delta T_{\frac{1}{2}}$ values of the narrow and broad components of the endotherms were similar to those seen with the DOPC-cholesterol and DSPC-cholesterol mixtures. There was very little change in the $\Delta T_{\frac{1}{2}}$ values of the narrow components, but the $\Delta T_{\frac{1}{2}}$ values of the broad components increased as the cholesterol concentration increased.

D. Effects of Cholesterol on DPPC

To determine if the results of the calorimetric analyses and the deconvolution of the endotherms used here were comparable with those reported before for other lipid-cholesterol mixtures (Estep et al., 1978, 1979, 1981; Mabrey et al., 1978) the thermotropic behavior of mixtures of cholesterol with DPPC was studied. Figure III-19 shows the effects of cholesterol on the enthalpies associated with the narrow components of the endotherms for DPPC-cholesterol obtained using the deconvolution described in this work.

Figure III-19

The enthalpies associated with the narrow components of the endotherms obtained on heating dispersions of mixtures of cholesterol with DPPC. (○) data from Estep *et al.* (1978); (·····) linear least squares fit to the data of Estep *et al.* (1978); (□) data from Mabrey *et al.* (1978); (– – –) linear least squares fit to the data of Mabrey *et al.* (1978); (△) data obtained in the course of this work; (——) linear least squares fit to this data.


8·0
6·0
4·0
2·0
0
0·1
0·2
ΔH (narrow comp. in kcal·mol⁻¹)
X cholesterol

The plots obtained by Estep et al., (1978) and Mabrey et al. (1978) for DPPC-cholesterol are also shown. My results agree well with those of Mabrey et al. (1978) and are close to those of Estep et al. (1978).

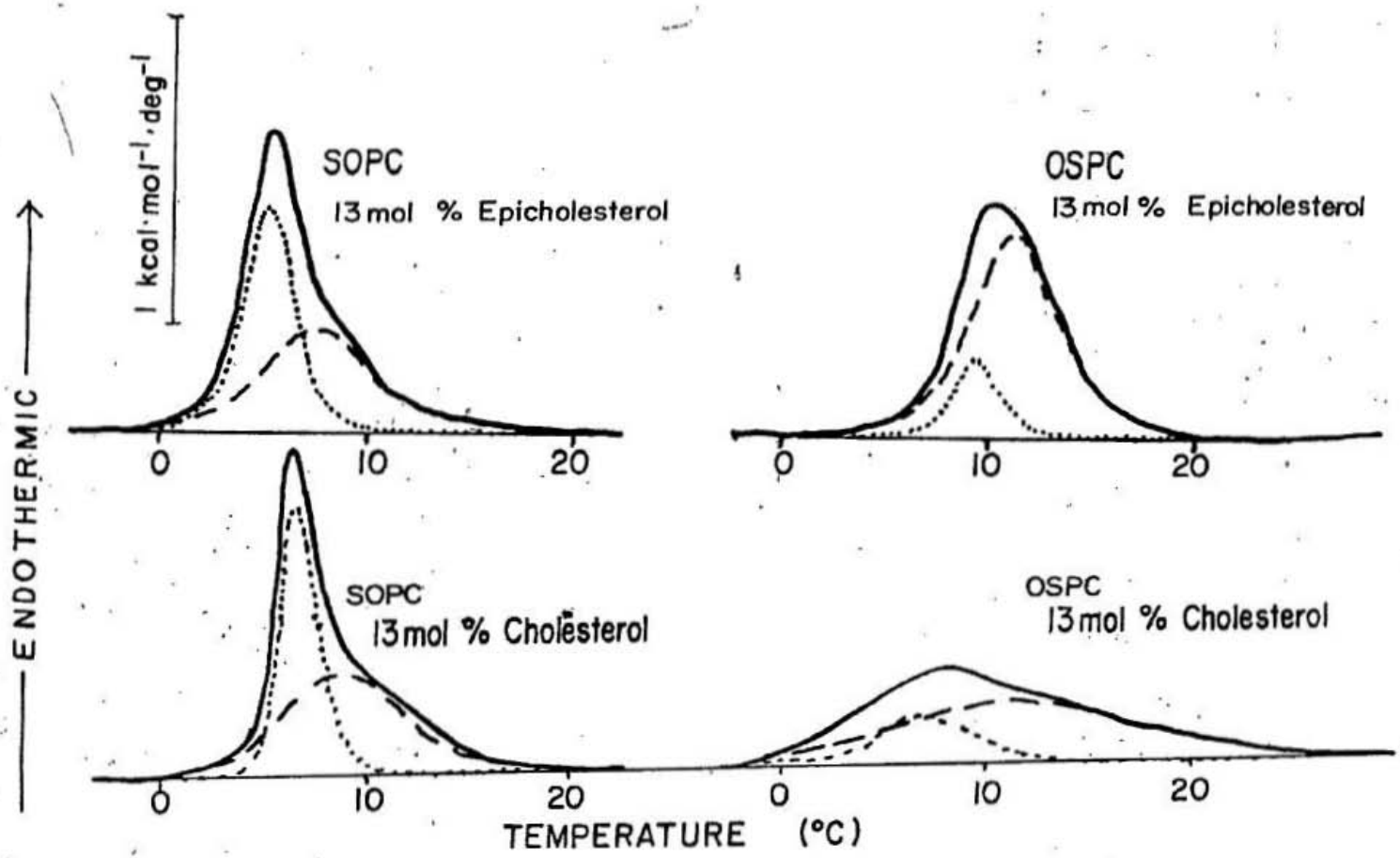
III-4 Effects of Epicholesterol on OSPC and SOPC

It was suggested by Huang (1977) that cholesterol might interact differently with positional isomers of saturated-unsaturated mixed-acid lecithins. The model of Huang (1977) involved the formation of hydrogen bonds between the 3-β hydroxyl of cholesterol and specific carbonyl oxygens at the sn-1 position of the lecithin. If the 3-β hydroxyl group was required for the differential effects of cholesterol on the different positional isomers, these differences in the effects of cholesterol would be decreased or completely abolished by the loss of the β-hydroxyl group. To determine the consequences, if any, of the absence of the β-hydroxyl on the effect of cholesterol on the positional isomers, mixtures of epicholesterol (the 3α-hydroxy epimer of cholesterol) with OSPC and SOPC were studied.

Figure III-20 shows the normalized heating endotherms obtained for mixtures of epicholesterol with OSPC and SOPC at $X_{epichol} = 0.13$ and the components of the endotherms obtained using the deconvolution described before. For comparison, endotherms for corresponding mixtures of cholesterol with OSPC and SOPC are also shown. The shapes of the endotherms for the mixtures with epicholesterol are

Figure III-20

The effects of 13mol% cholesterol or 13mol% epi-cholesterol on the heating endotherms of SOPC or OSPC. (——) composite endotherms, (·····) narrow components of the endotherms, and (– – –) broad components of the endotherms obtained using the computer analysis described in Section II-7E.


1 kcal·mol⁻¹·deg⁻¹
SOPC
13 mol % Epicholesterol
OSPC
13 mol % Epicholesterol
SOPC
13 mol % Cholesterol
OSPC
13 mol % Cholesterol
ENDOTHERMIC
0
10
20
TEMPERATURE (°C)

very similar to those for mixtures with cholesterol. The epicholesterol caused a greater reduction in the maximum excess heat capacity of the phase transition for OSPC than for SOPC.

The resolved components of the endotherms for the mixtures with epicholesterol were also similar to those for the corresponding mixtures with cholesterol. The broad components had higher T_{max} values than the narrow components. The enthalpies for the transitions and the enthalpies attributable to the components of the endotherms for the mixtures with epicholesterol and with cholesterol are given in Table III-7. The total enthalpies of the transitions were lower for the cholesterol mixtures than for the epicholesterol mixtures. This was consistent with the reports that epicholesterol had less effect on liposome permeability than cholesterol (de Kruyff et al., 1972, 1973) and caused less condensation of lecithins in monolayers (Demel et al., 1972; Ghosh and Tinoco, 1972). The smaller effects of epicholesterol on the transition enthalpies relative to those of cholesterol notwithstanding, the differences between the effects of epicholesterol on the OSPC and the effects on SOPC were similar to those observed with mixtures of cholesterol and these lecithin isomers. The narrow component of the endotherms of OSPC-epicholesterol had a lower enthalpy than the narrow component of SOPC-epicholesterol and the enthalpy of the broad component of OSPC-epicholesterol was greater than that of SOPC-epicholesterol.

TABLE III-7

Thermotropic Data From Thermograms of Positional Isomers of PCs Mixed With 13 mol% Cholesterol and Epicholesterol

LIPID	STEROYL	ΔH_{total} ($kcal \cdot mol^{-1}$)	$\Delta H_{narrow\ comp}$ ($kcal \cdot mol^{-1}$)	$\Delta H_{broad\ comp.}$ ($kcal \cdot mol^{-1}$)
SOPC	Cholesterol	†5.5	†2.4	†3.1
	Epicholesterol	7.5	3.8	3.7
OSPC	Cholesterol	*3.8	*0.7	*2.9
	Epicholesterol	5.2	0.8	4.4

†Average of three samples

*Average of two samples

Epicholesterol reduced total enthalpy by a smaller amount than did cholesterol for both lipids. This was found before by Dr. Kruyff et al. (1972).

Epicholesterol acted in a fashion very similar to cholesterol in that it had a more profound effect in the narrow component of OCPS-epicholesterol than in SOPC-epicholesterol.

III.5 Binary Mixtures of Lecithins

Bilayers of binary mixtures of saturated single-acid lecithins have been studied before (Phillips et al., 1970; Shimshick and McConnell, 1973; Chapman et al., 1974; Mabrey and Sturtevant, 1976; vanDijck et al., 1977). It was shown that lecithins with acyl chains of almost equal carbon number form near ideal mixtures in bilayers and that the greater the difference on the acyl chains of the lecithins, the less ideal was the mixing, especially in the gel phase (Mabrey and Sturtevant, 1976). Attempts have been made to quantitate the non-ideality of mixing of lecithins in binary systems (Lee, 1977; Cheng 1979; vonDreele, 1980; Freire and Snyder, 1980). Applying "Regular Solution Theory" (Hildebrand and Scott, 1964), Lee (1977) obtained estimates of the excess energy of interaction (ρ_o) of unlike molecules in a binary mixture. These excess interaction energies represent the difference in the interaction energies between like pairs of molecules and unlike pairs. A positive value of ρ_o indicates that additional energy is required to form an unlike pair from two like pairs and so there is a greater tendency for the formation of clusters of like molecules.

In this work the effects of small structural changes in one acyl chain of mixed-acid lecithins on the excess interaction energy of these lecithins with some single-acid lecithins have been investigated in aqueous dispersions of binary lipid mixtures. Using the approach of Lee (1977)

attempts have been made to quantitate the excess interaction energies in the systems studied here. Knowledge of the effects of the structure of acyl chains on the mixing of lecithins could be very useful in predicting the molecular organization of lipids and the existence of lateral phase separation in membranes and in pulmonary surfactant.

A. Mixing of DPPC with POPC and SOPC

A series of binary mixtures of DPPC with two mixed-acid lecithins that had an oleate chain at the sn-2 position and saturated chains of sixteen (palmitate) or eighteen (stearate) carbons at the sn-1 position were studied by differential scanning calorimetry. DPPC was chosen as the second component in these mixtures because the behavior of DPPC in bilayers has been better characterized than that of any other lecithin. The choice of the 1-saturated-2-unsaturated mixed-acid lecithins reflected the common occurrence of these lecithins in biological systems.

1. Calorimetry and the Construction of the Phase Diagrams

Heating endotherms obtained for some aqueous dispersions of mixtures of DPPC with POPC and SOPC are shown in Figure III-21. The dsc scans of the DPPC-POPC mixtures were carried out by Dr. K. P. Coolbear. The endotherms of the dispersions of POPC containing 20 to 70mol% of DPPC and of SOPC containing 18 to 84mol% of DPPC were broad and almost

Figure III-21

Endotherms obtained on heating aqueous dispersions of mixtures of DPPC with POPC (A), and with SOPC (B).

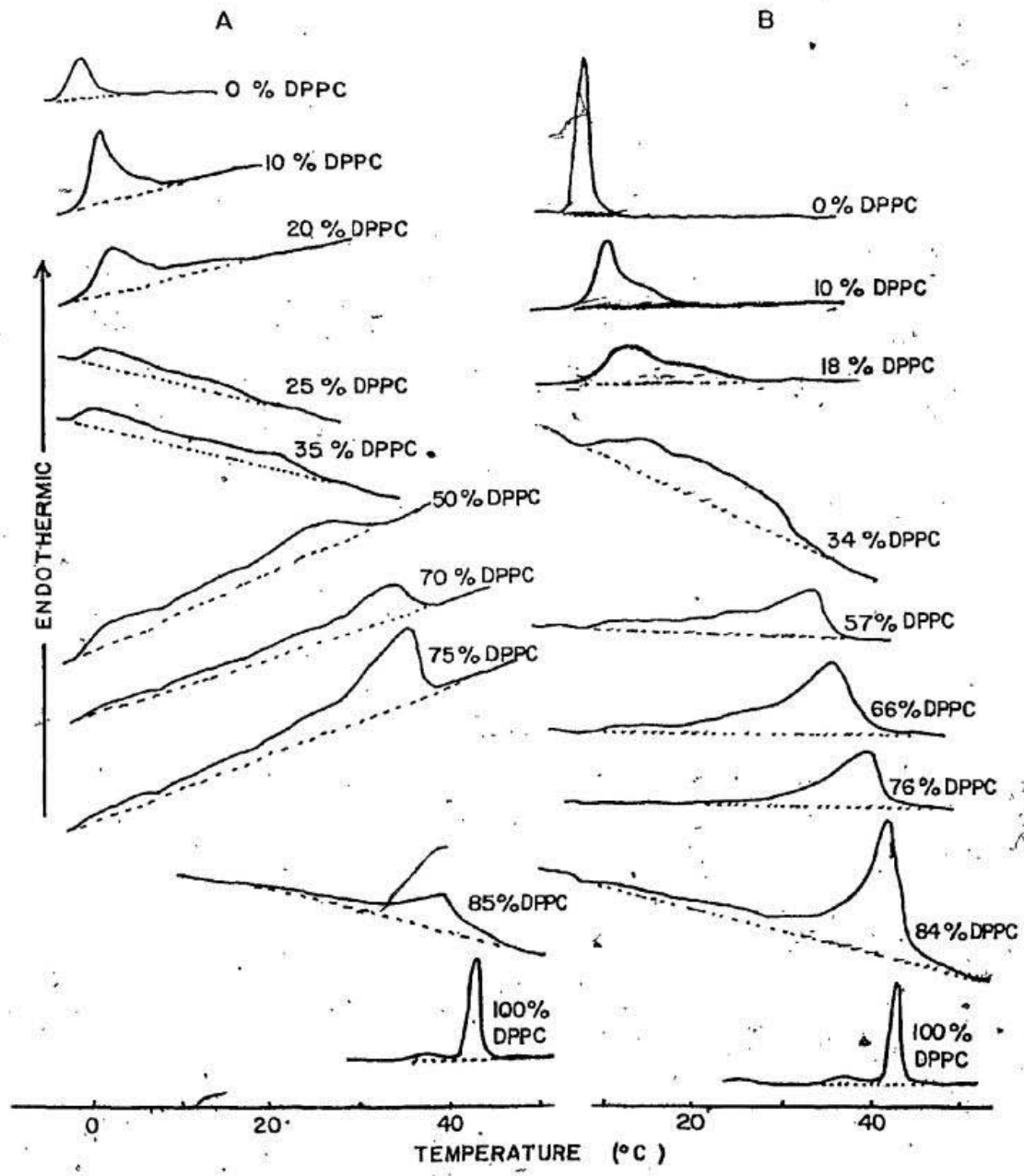

A
B
0 % DPPC
10 % DPPC
20 % DPPC
25 % DPPC
35 % DPPC
50 % DPPC
70 % DPPC
75 % DPPC
85% DPPC
100% DPPC
0% DPPC
10 % DPPC
18 % DPPC
34 % DPPC
57% DPPC
66% DPPC
76% DPPC
84 % DPPC
100 % DPPC
ENDOTHERMIC
0
20
40
20
40
TEMPERATURE (°C)

Figure III-22

Phase diagram for mixtures of DPPC with POPC. (○) liquidus temperatures, (●) solidus temperatures, and (——) curves computed for ideal mixing.

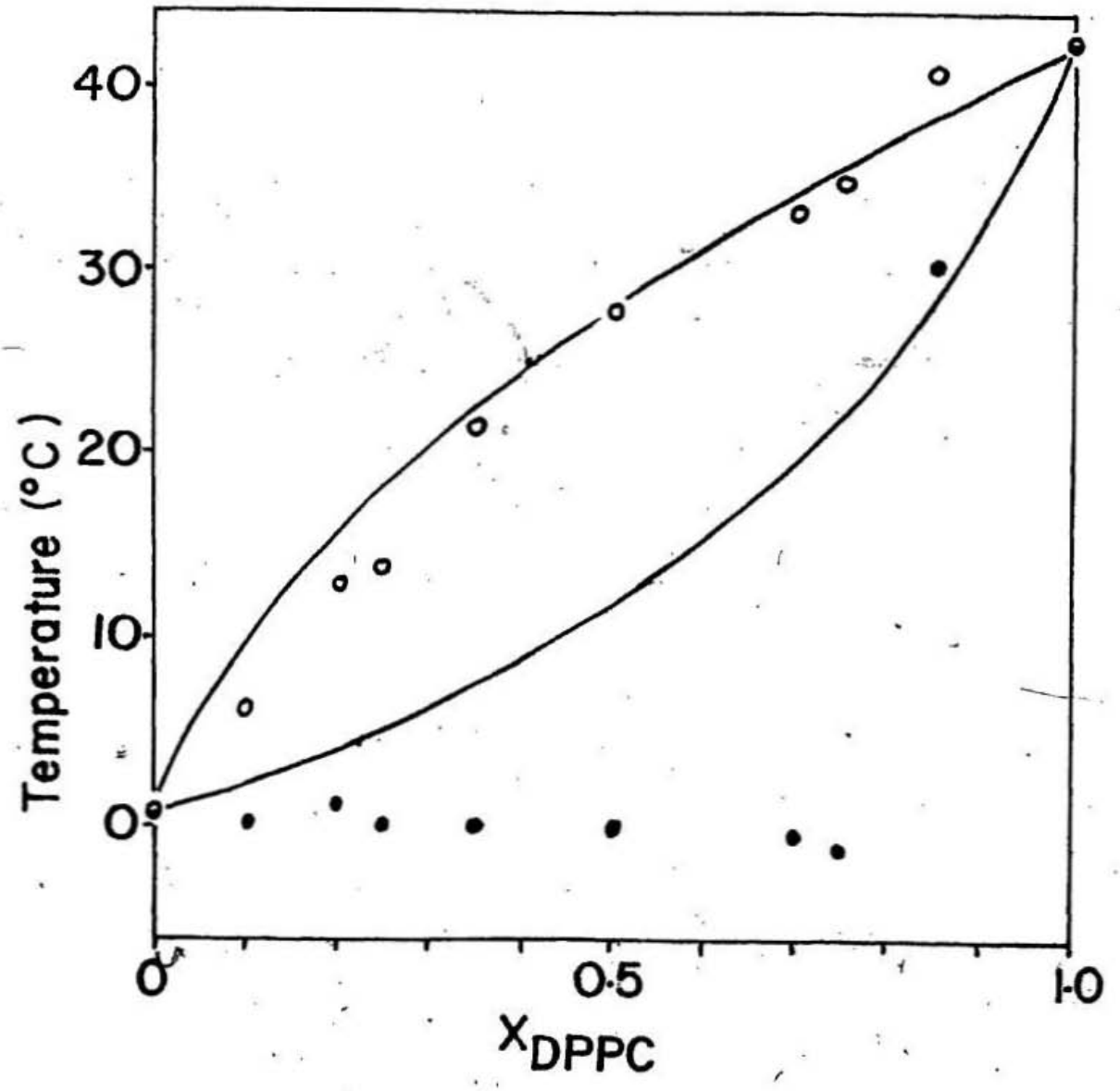
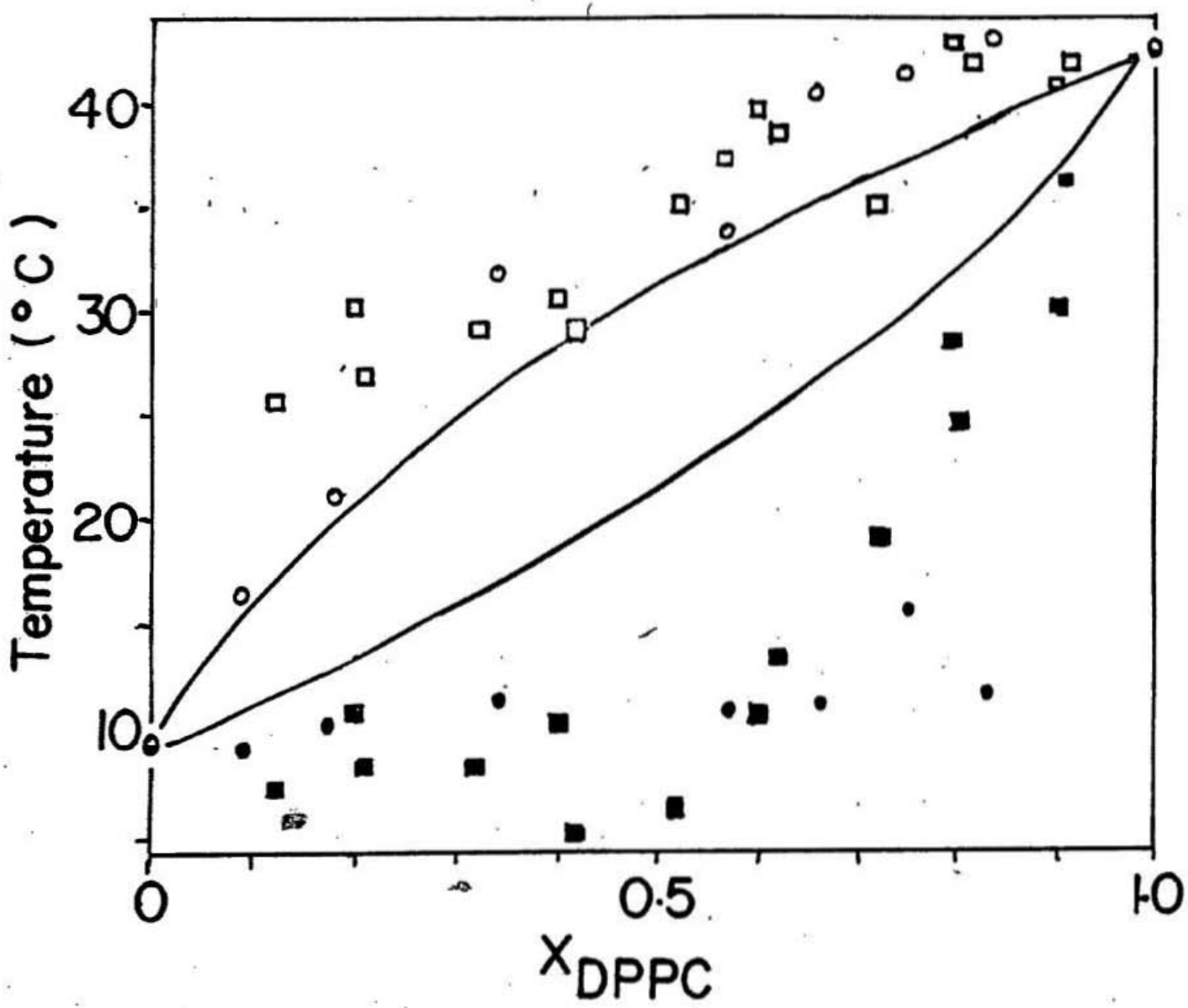

40
30
20
10
0
Temperature (°C)
0
0·5
1·0
X_{DPPC}

Figure III-23

Phase diagram for mixtures of DPPC with SOPC. Liquidus temperatures (□: 6% acyl migrated SOPC; 0:18% acyl migrated SOPC) and solidus temperatures (■: 6% acyl migrated SOPC; ●:18% acyl migrated SOPC), and (——) liquidus and solidus curves computed for ideal mixing.


Temperature (°C)
40
30
20
10
0
0·5
1·0
X_{DPPC}

bimodal in appearance.

Phase diagrams were constructed from the dsc data and these are shown in Figures III-22 and III-23. The transition temperatures used to construct these phase diagrams were the temperatures at which the excess heat capacity curves depart from the extrapolated baselines of the heating (solidus) and cooling (liquidus) dsc scans. Because the presence of reversed positional isomers might affect the mixing behavior of the mixed-acid lecithins, mixtures of DPPC with SOPC containing 6mol% of OSPC and SOPC containing 18mol% of OSPC were studied. No substantial differences in the behavior of these two sets of mixtures were observed (Figure III-23). In both phase diagrams, the solidus temperatures appeared to be independent of the concentrations of DPPC up to about 50mol% of DPPC. This behavior suggested substantial immiscibility of the two components in the gel phase in both mixtures. Phase diagrams calculated assuming ideal mixing of components in both the gel and the liquid-crystal are also shown in Figure III-22 and III-23. For both pairs of lecithins the observed liquidus data were close to the ideal liquidus curves. The solidus data were very different from the curve predicted for ideal mixing in the gel phase for both pairs of lecithins.

ii. Estimation of the Non-Ideality of Mixing

Although visual inspection of the phase diagrams provides some idea of the mixing behavior of the components in

binary mixtures, the information is strictly qualitative. Any attempts to evaluate differences in the mixing behavior of components in different mixtures requires a quantitative estimate of the non-ideality of mixing.

The initial attempts to obtain estimates of the excess interaction energies for the liquidus (ρ_o^{LIQ}) and solidus (ρ_o^{SOL}) phases for the two binary mixtures were carried out by iteratively fitting the experimental data to the equation of Lee (1977) using the Method I (Section II-7E). Initial estimates were made of the values of ρ_o^{LIQ} and ρ_o^{SOL}. These were used in Equation [II-1] and the ρ_o^{LIQ} and ρ_o^{SOL} were adjusted to obtain best fits to the data . The values of ρ_o^{LIQ} and ρ_o^{SOL} that gave this best fit were used as initial estimates in Equation II-2. Again ρ_o^{LIQ} and ρ_o^{SOL} were adjusted to fit Equation II-2 to the data and the resulting values were used as the initial guesses for fitting Equation II-1. Repetition of this procedure should have resulted in convergence of the ρ_o estimates from the two equations. The ρ_o^{LIQ} and ρ_o^{SOL} values obtained in this procedure for the DPPC-POPC and DPPC-SOPC mixtures did not converge suggesting that no unique values of ρ_o^{LIQ} and ρ_o^{SOL} existed which would fit the data to both equations, or that the assumptions in the derivation of equations II-1 and II-2 were not valid.

The approach of Lee (1977) that gives rise to Equations II-1 and II-2 was based on the assumption that the two components of the mixtures are miscible in both the gel and the

liquid-crystal, but the DPPC-POPC and DPPC-SOPC phase diagrams suggested that these pairs of lecithins might be immiscible in the gel.

If the two components of a binary mixture are completely immiscible in the gel, the excess interaction energy of the two lipids in the liquid-crystal can be described by Equation II-3 (Lee, 1977). The data from the two phase diagrams were fitted to this equation and estimates of the excess interaction energies of unlike molecules in the liquid-crystalline phase were obtained. The p_o^{LIQ} value for the mixtures of DPPC with POPC was 0.09 kcal $\cdot$ mol^{-1} and for the mixtures of DPPC with SOPC the value was 0.46 kcal$\cdot$mol^{-1}. The solidus and liquidus curves computed using these p_o^{LIQ} values are shown in Figures III-24 and III-25 and the standard deviations of the residuals of temperature for these fitted curves are given in Table III-8.

B. Mixing of DMPC with PSPC and POPC.

As was mentioned before, differences in the acyl chain length of saturated single-acid lecithins affected their mixing behavior in binary systems. To investigate the effects of changes in the length of the acyl chains of the saturated single-acid lecithins on their mixing with saturated-unsaturated mixed-acid lecithins, mixtures of DMPC with POPC were studied.

The effects of the introduction of a double bond on the

Figure III-24

Phase diagram for mixtures of DPPC with POPC. (○) liquidus temperatures; (●) solidus temperatures; (——) curves computed assuming immiscibility in the gel and a p_o^{LIQ} of 0.09 kcal·mol^{-1}.

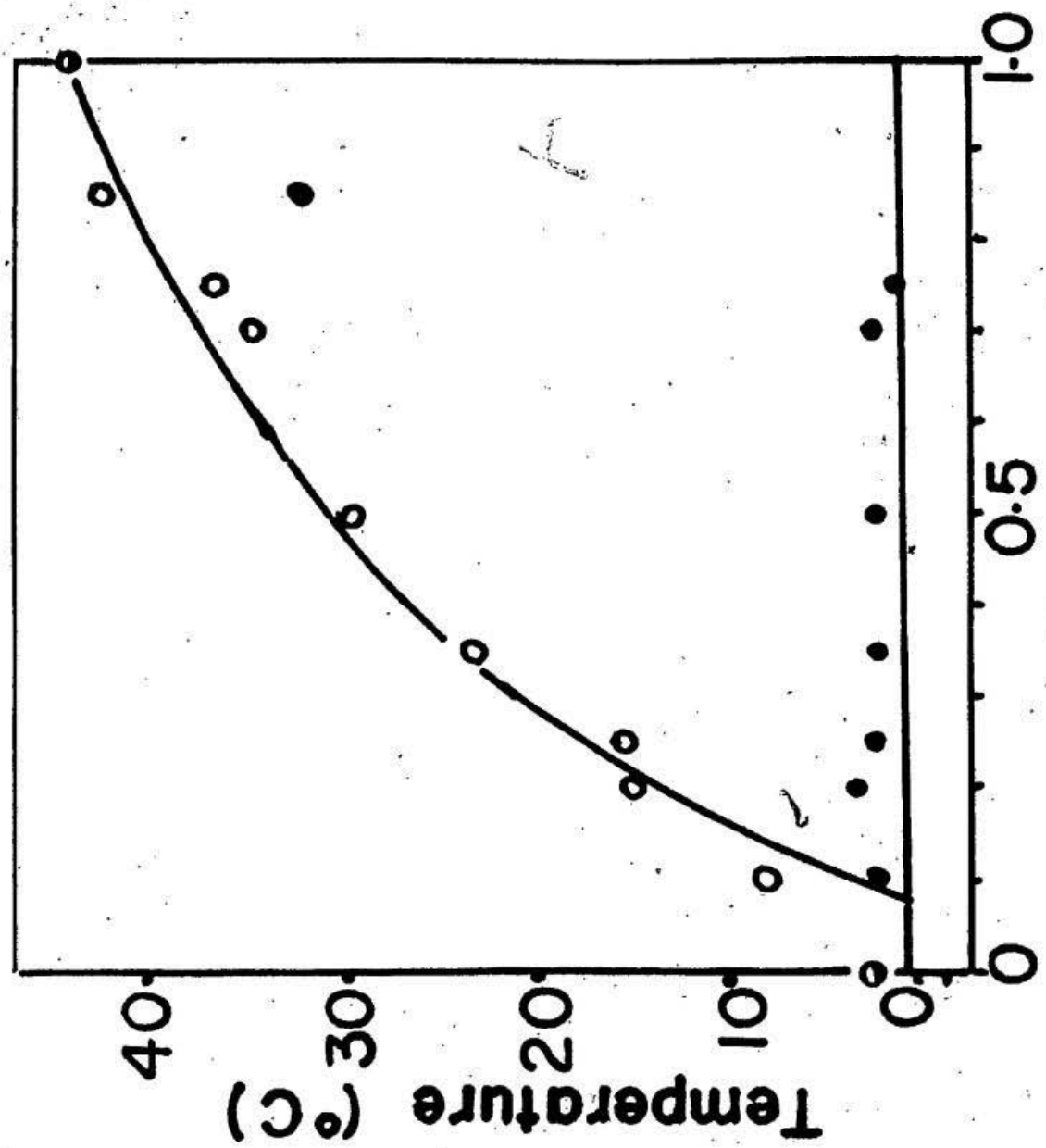

Temperature (°C)
40
30
20
10
0
0
0·5
1·0
X_{DPPC}

Figure III-25

Phase diagram for mixtures of DPPC with SOPC. Liquidus temperatures (□: 6% acyl migrated SOPC; 0+18% acyl migrated SOPC), solidus temperatures (■: 6% acyl migrated SOPC; ●: 18% acyl migrated SOPC), and (——) curves computed assuming immiscibility in the gel and $\rho_o^{LIQ} = 0.46$ kcal·mol^{-1}.

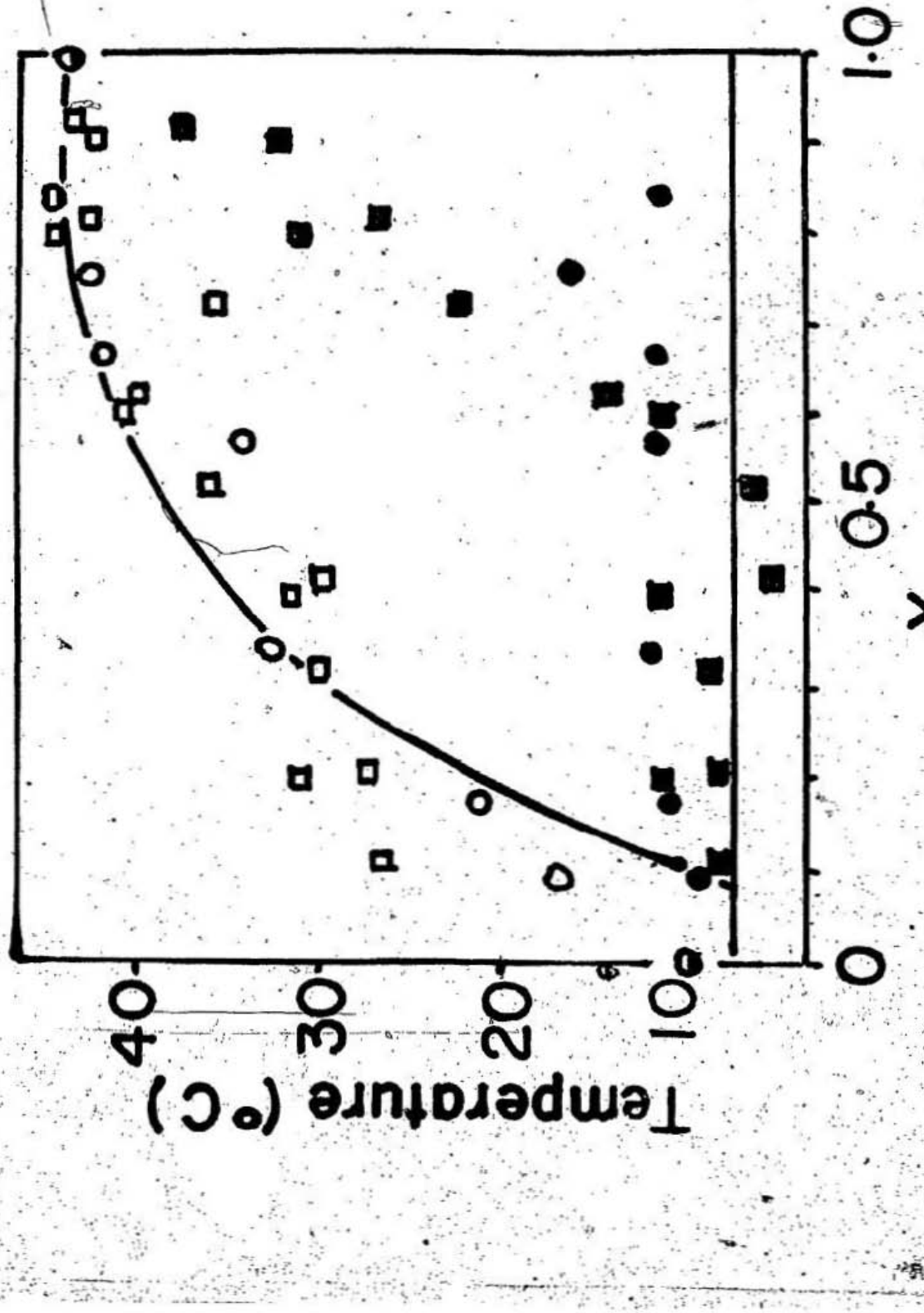

Temperature (°C)
40
30
20
10
0
0·5
1·0
X_{DPPC}

TABLE III-8

A. ρ_o^{LIQ} values for POPC-DPPC and for SOPC-DPPC mixtures by fitting to equation for gel state immiscibility*

Lipids	ρ_o^{LIQ}	†MS
POPC-DPPC	0.09 kcal·mol^{-1}	2.8 degrees
SOPC-DPPC	0.43 kcal·mol^{-1}	5.8 degrees

B. ρ_o^{LIQ} and ρ_o^{SOL} for POPC-DMPC and for PSPC-DMPC mixtures obtained by Method II (Section II-7E)

Lipids	ρ_o^{LIQ} (kcal·mol^{-1})	†MS^{LIQ} (degrees)	ρ_o^{SOL} (kcal·mol^{-1})	†MS^{SOL} (degrees)
POPC-DMPC	0.27	1.9	0.67	2.3
PSPC-DMPC	0.76	1.7	1.01	1.8

C. ρ_o^{LIQ} and ρ_o^{SOL} for POPC-SMPC and for PSPC-DMPC mixtures obtained using Method III (Section II-7E)

Lipids	ρ_o^{LIQ} (kcal·mol^{-1})	†MS^{LIQ} (degrees)	ρ_o^{SOL} (kcal·mol^{-1})	†MS^{SOL} (degrees)
POPC-DMPC	0.23	1.4	0.42	1.7
PSPC-DMPC	0.70	1.2	0.90	2.0

* MS of residuals of Temperature for solidus curves were 12.4 and 13.2 degrees respectively for POPC-DPPC and the SOPC-DPPC.

†MS is the mean square of the residuals of temperature.

mixing of a mixed-acid lecithin with a saturated single-acid lecithin were also investigated by the study of the mixtures of DMPC with PSPC and the mixtures of DMPC with POPC. It is noteworthy that the differences in the transition temperatures between the two lecithins in each of these binary mixtures were almost equal. The difference in T_c between DMPC and PSPC was 24°C and the difference in T_c between DMPC and POPC was 26°C.

i. Calorimetry and Construction of Phase Diagrams

Typical endotherms obtained on heating mixtures of DMPC with PSPC and DMPC with POPC are shown in Figure III-26. It is noteworthy that pretransition endotherms normally seen with pure DMPC and pure PSPC were observed in the scans of DMPC containing up to 30mol% PSPC and in scans of DMPC having 80 and 90mol% PSPC. Mixtures containing 40 and 50mol% PSPC did not exhibit pretransition endotherms. No pretransitions were observed in the scans of mixtures of DMPC with POPC.

The phase diagrams constructed for mixtures of DMPC with PSPC and with POPC are shown in Figure III-27 and III-28. The shapes of the solidus curves in these phase diagrams suggested that gel state immiscibility occurred in only a small composition range of each phase diagram (0 to 10mol% of DMPC in POPC and 0 to 30mol% of PSPC in DMPC). In this respect, these mixtures were substantially different from the mixtures of DPPC with POPC and with SOPC. The

Figure III-26

Endotherms obtained on heating aqueous dispersions of mixtures of DMPC with POPC (A) and with PSPC (B).

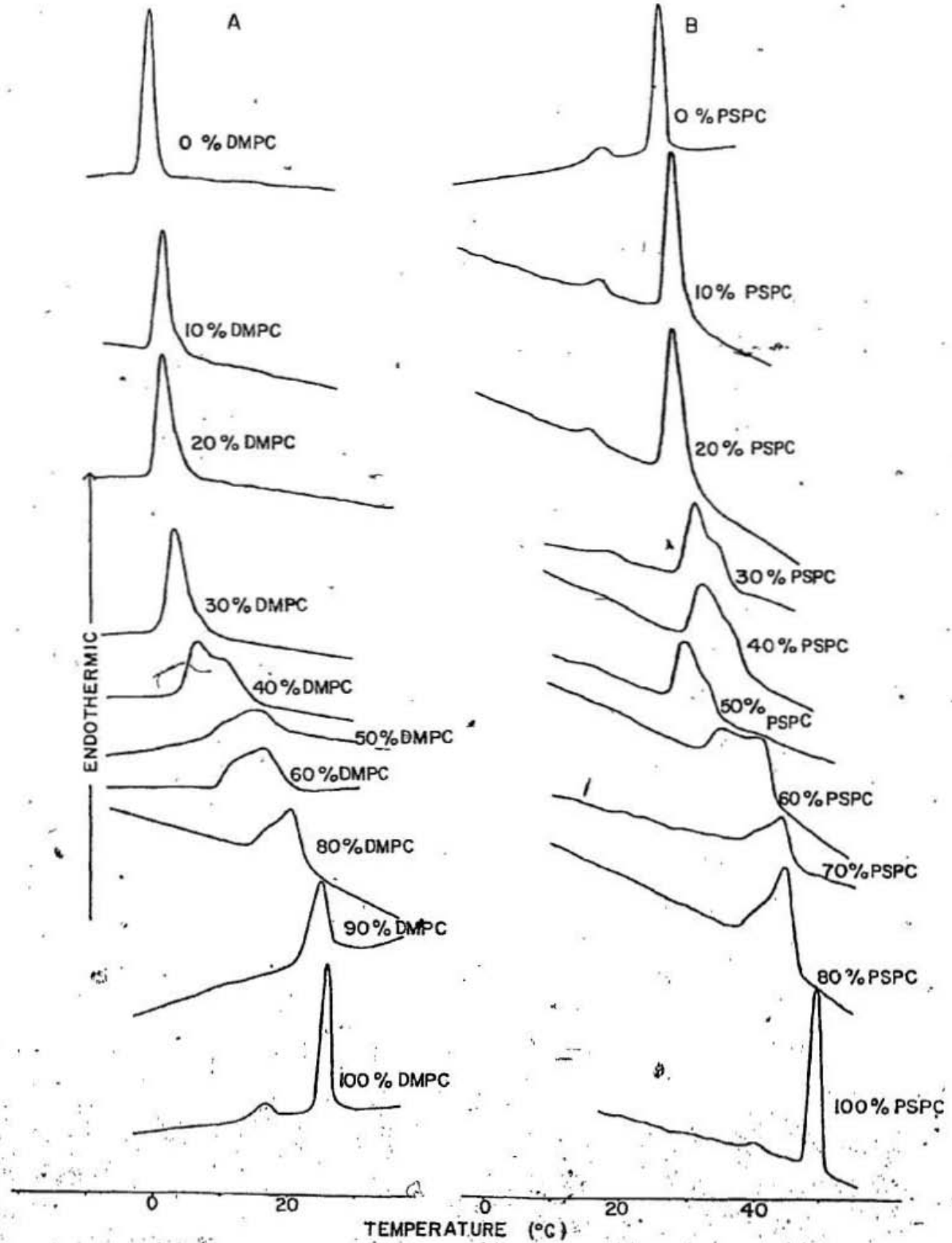

A
0 % DMPC
10 % DMPC
20 % DMPC
30 % DMPC
40 % DMPC
50 % DMPC
60 % DMPC
80 % DMPC
90 % DMPC
100 % DMPC
B
0 % PSPC
10 % PSPC
20 % PSPC
30 % PSPC
40 % PSPC
50 % PSPC
60 % PSPC
70 % PSPC
80 % PSPC
100 % PSPC
ENDOTHERMIC
0
20
0
20
40
TEMPERATURE (°C)

Figure III-27

Phase diagram for mixtures of DMPC with PSPC. (○) liquidus temperatures, (●) solidus temperatures, and (——) liquidus and solidus curves computed for ideal mixing.

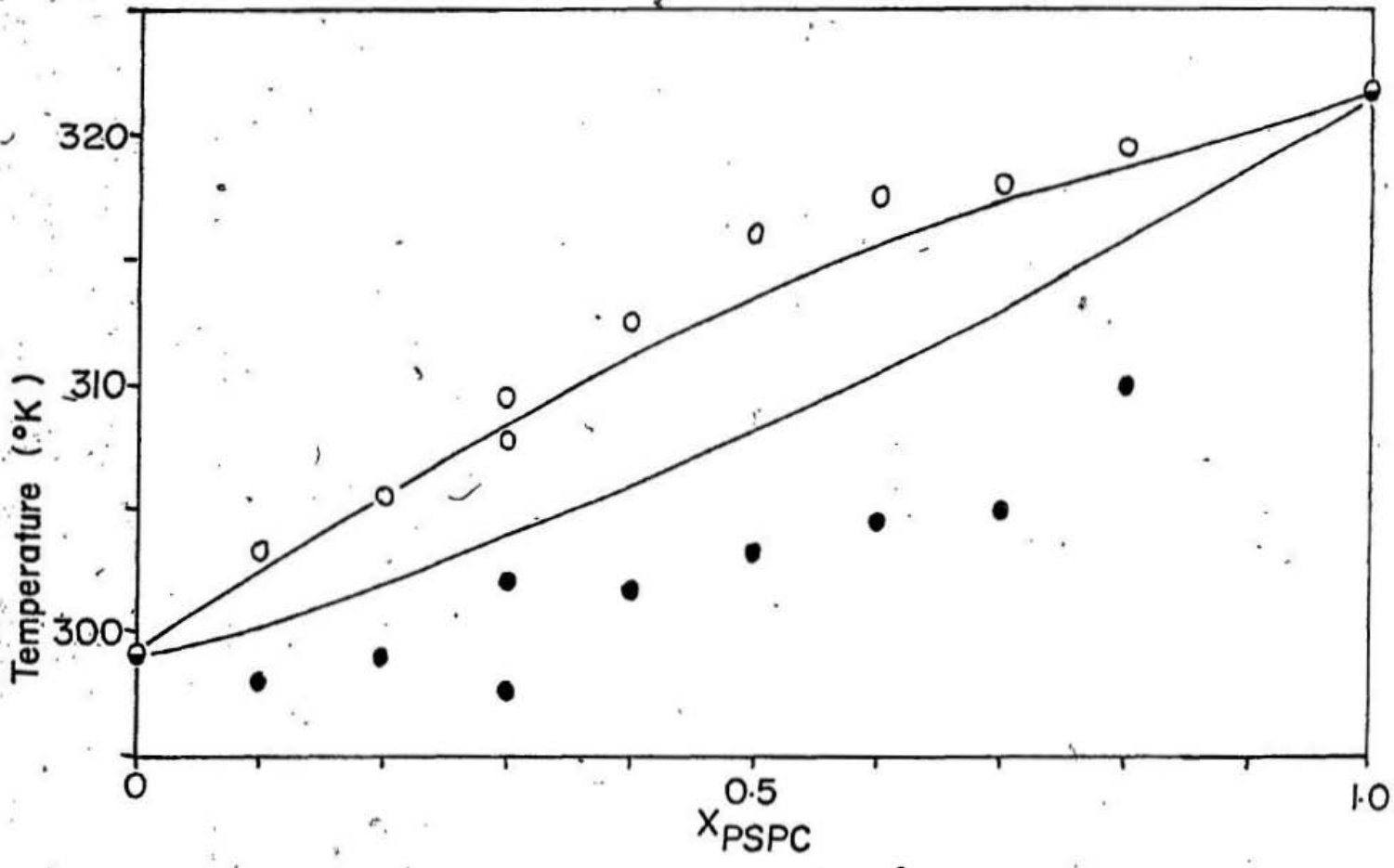
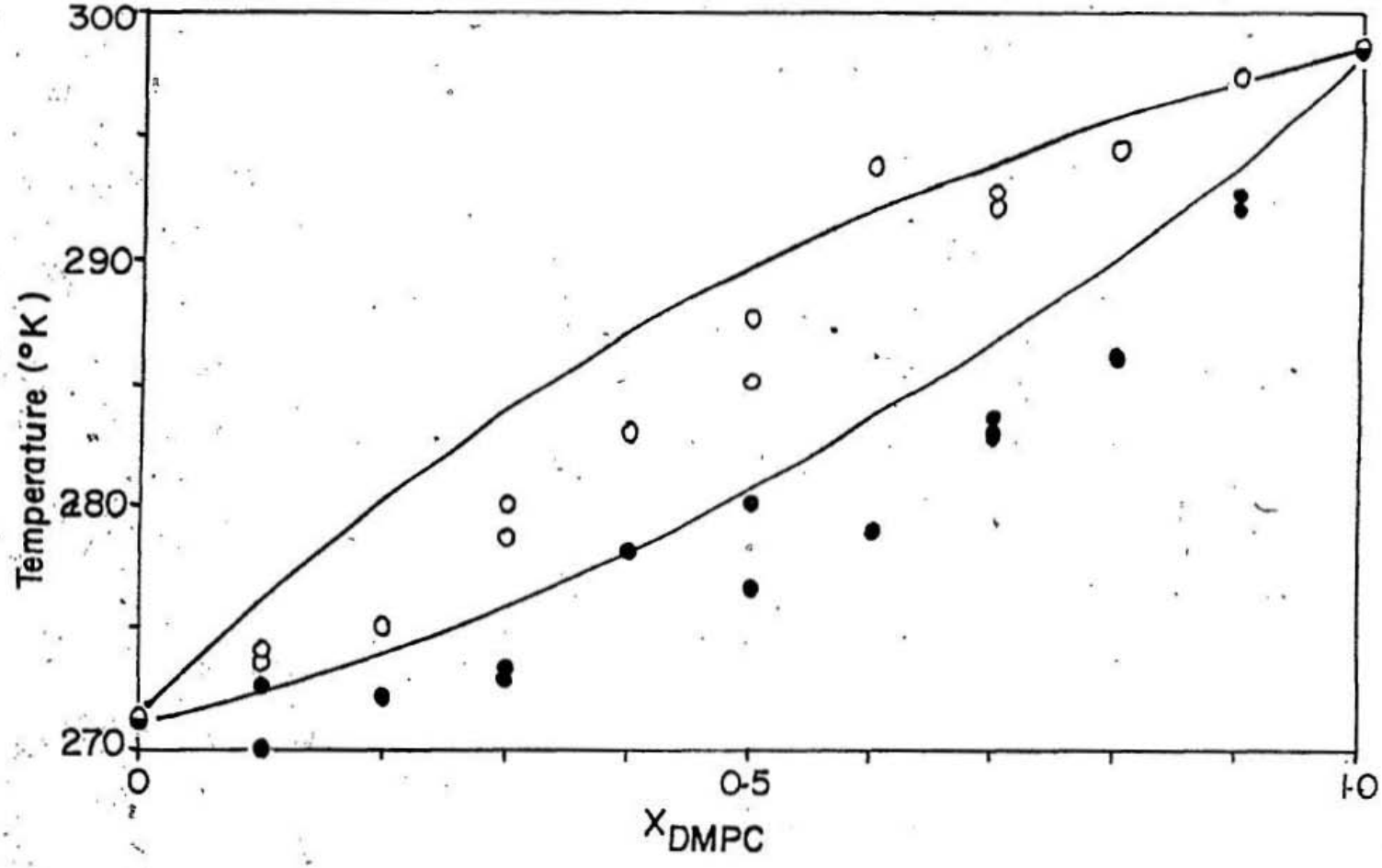

320
310
300
Temperature (°K)
0
0·5
1·0
X_{PSPC}

Figure III-28

Phase diagram for mixtures of DMPC with POPC. (○) liquidus temperatures, (●) solidus temperatures, and (——) liquidus and solidus curves computed for ideal mixing.


300
290
280
270
Temperature (°K)
0
0·5
1·0
X_{DMPC}

solidus and liquidus curves computed for ideal mixing in both phases of DMPC with PSPC and with POPC are shown in Figures III-27 and III-28.

ii. Estimation of the Non-Ideality of Mixing

Attempts were made to estimate ρ_o^{LIQ} and ρ_o^{SOL} by iteratively fitting the DMPC-PSPC and DMPC-POPC data to the two equations (Equations II-1 and II-2) of Lee (1977). For both binary mixtures the ρ_o estimates obtained from the two equations did not converge on unique values. It was unlikely, in view of the shapes of the DMPC-PSPC and DMPC-POPC phase diagrams, that any extensive immiscibility in the gel state occurred with these two binary mixtures. For this reason the use of Equation II-3 to obtain estimates of ρ_o^{LIQ} by assuming gel state immiscibility did not seem justified.

Equations II-1 and II-2 can be solved simultaneously for any values of temperature (T) throughout the phase diagram. For each T, the values of X_A^{LIQ} and X_A^{SOL} are the values of X_A at which a line through T and parallel to the X-axis intersects the liquidus and solidus curves, respectively. Simultaneous solution of the two equations for each set of T, X_A^{LIQ} and X_A^{SOL} values yields a series of ρ_o^{LIQ} and ρ_o^{SOL}, values. Since the excess interaction energies are dependent on the nature of the interaction between unlike pairs and between like pairs of molecules it would be expected that these parameters would be independent of the temperature and composition of the mixture. One would

expect the ρ_o values obtained by simultaneous solutions of Equations II-1 and II-2 at different points on the phase diagram to be equal within the error limits of the estimates.

The ρ_o^{LIQ} and ρ_o^{SOL} values obtained by simultaneous solution of the two equations at different points on the DMPC-PSPC phase diagram are shown in Figure III-29. The ρ_o values for the DMPC-POPC phase diagram obtained using this procedure are shown in Figure III-30. For both binary mixtures the ρ_0 values estimated in this way were not constant over the entire composition, but were high at the extremes. Since errors in the determination of temperature from the dsc thermograms and errors in the composition of the mixtures could introduce errors in the ρ_o estimates, an effort was made to evaluate the potential errors in ρ_o occurring because of possible measurement errors in T, X_A^{LIQ} or X_A^{SOL}. The nature of the two equations results in the sensitivity of ρ_o values to changes in T or X_A being greater at the extremes of composition. This effect is seen in Table III-9 which shows the errors in ρ_o that would result from potential measurement errors in T of $\pm$ 0.5 degrees and errors in X_A of $\pm$ 0.005. These values were chosen as conservative estimates of the errors in my measurements of T and X_A. It is noteworthy that duplicate samples used to construct the phase diagrams show variations in T of up to $\pm$ 2 degrees. The results in Table III-9 indicate that the errors in ρ_o values arising from errors in measurement of T were

Figure III-29

Variation in the estimates of ρ_o^{LIQ} and ρ_o^{SOL} obtained by simultaneous solution of the equations of Lee (1977), with the composition of the mixture for mixtures of DMPC and PSPC. Error bars are the potential errors in ρ_o that would arise because of potential errors of $\pm$ 0.5 degree in the measurement of the transition temperatures; (○) ρ_o^{LIQ}; (●) ρ_o^{SOL}.

2·0
1·0
0
0·5
1·0
ζ (kcal · mol^{-1})
x^{liq}_{PSPC}

Figure III-30

Variation in the estimates of p_o^{LIQ} and p_o^{SOL}, obtained by simultaneous solution of the equations of Lee (1977), with the composition of the mixtures for mixtures of DMPC with POPC. Error bars are the errors in p_o that would result from a potential error of $\pm$ 0.5 degree in the measurement of temperature; (○): p_o^{LIQ}; (●): p_o^{SOL}.

ΔG_{ex} (kcal·mol^{-1})
2·0
1·0
0·0
0·0
0·5
1·0
X^{liq}_{DMPC}

TABLE III-9

Error in ρ_o estimates resulting from potential measurement errors in temperature or composition at the extremes and center of POPC/DMRC phase diagram.

		Calculated values for ρ_o		Errors occuring in ρ_o estimates because of potential measurement in T of ±0.5 degrees	
X_o^{LIQ}	T (°K)	ρ_o^{LIQ} (kcal·mol^{-1})	ρ_o^{SOL} (kcal·mol^{-1})	LIQ (kcal·mol^{-1})	SOL (kcal·mol^{-1})
0.11	274	0.47	0.81	±0.11	±0.18
0.43	285	0.25	0.56	±0.04	±0.00
0.87	295	0.18	0.80	±0.69	±0.53

		Calculated values for ρ_o		Errors occuring in ρ_o estimates because of potential measurement in X_A of ±0.005	
X_A^{LIQ}	T (°K)	ρ_o^{LIQ} (kcal·mol^{-1})	ρ_o^{SOL} (kcal·mol^{-1})	LIQ (kcal·mol^{-1})	SOL (kcal·mol^{-1})
0.11	274	0.47	0.81	±0.04	±0.02
0.43	285	0.25	0.56	±0.02	±0.01
0.87	295	0.18	0.80	±0.01	±0.13

substantially greater than those arising from errors in determining the composition of the mixtures. The error bars in Figures III-29 and III-30 represent the errors in ρ_o due to $\pm$ 0.5 degree errors in measurements of T. It appears that within the expected errors in evaluating the ρ_o values the ρ_o^{SOL} values obtained by the simultaneous solution of Equations II-1 and II-2 for both binary mixes were constant except at the extremes of composition. Since the shapes of the dsc thermograms for the mixtures in this region (high and low X_A) lead to increased potential measurement errors in T, the ρ_o obtained at the ends of the phase diagrams are likely to be the least reliable. The mean ρ_o values obtained for $0.2 < X_A < 0.8$ were evaluated for the two binary mixtures and the curves computed using these mean ρ_o values are shown in Figures III-31 and III-32.

Estimates of ρ_o^{LIQ} and ρ_o^{SOL} were also obtained by a fitting procedure in which the ρ_o values were varied until the computed curves from Equations II-1 and II-2 gave the best fit to the experimental data (see Method IV, Section II-7E). Best fits were taken as those for which the standard deviations of residuals of temperature were minimized. The ρ_o evaluated in this way are shown in Table III-8 and the curves computed for the DMPC-PSPC and DMPC-POPC phase diagram using these ρ_o are shown in Figures III-33 and III-34.

The ρ_o values obtained using either of the procedures

Figure III-31

Phase diagram for mixtures of DMPC with PSPC; (O) liquidus temperatures; (●) solidus temperatures; (——) liquidus and solidus curves computed for $P_o^{LIQ} = 0.76$ kcal·mol^{-1} and $P_o^{SOL} = 1.01$ kcal·mol^{-1}.

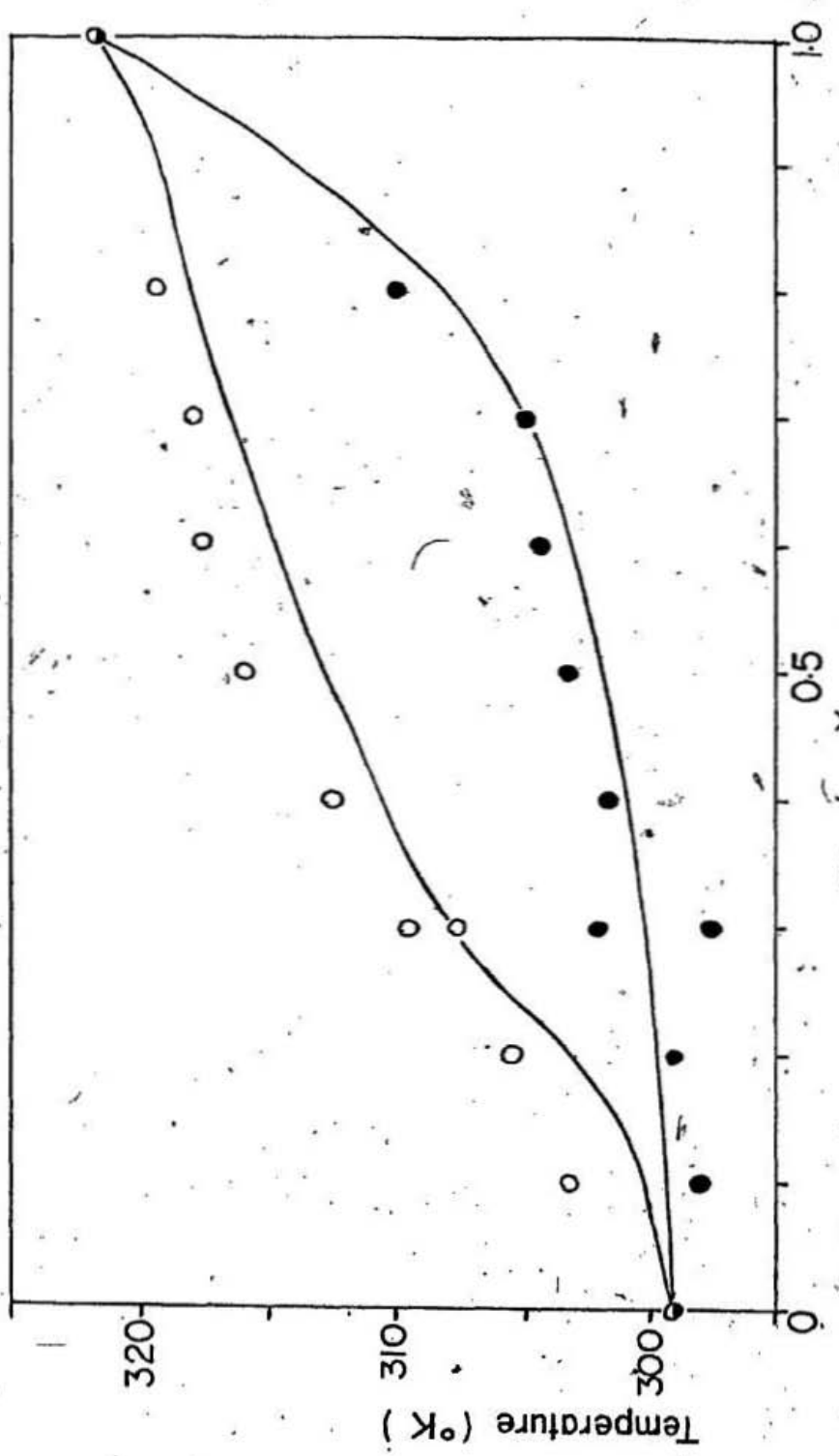

Temperature (°K)
320
310
300
0
0.5
1.0
X_{PSPC}

Figure III-32

Phase diagram for mixtures of DMPC with POPC. (○) liquidus temperatures; (●) solidus temperatures; (——) liquidus and solidus curves computed for $p_o^{LIQ} = 0.27$ kcal·mol-1 and $p_o^{SOL} = 0.62$ kcal·mol^{-1}.

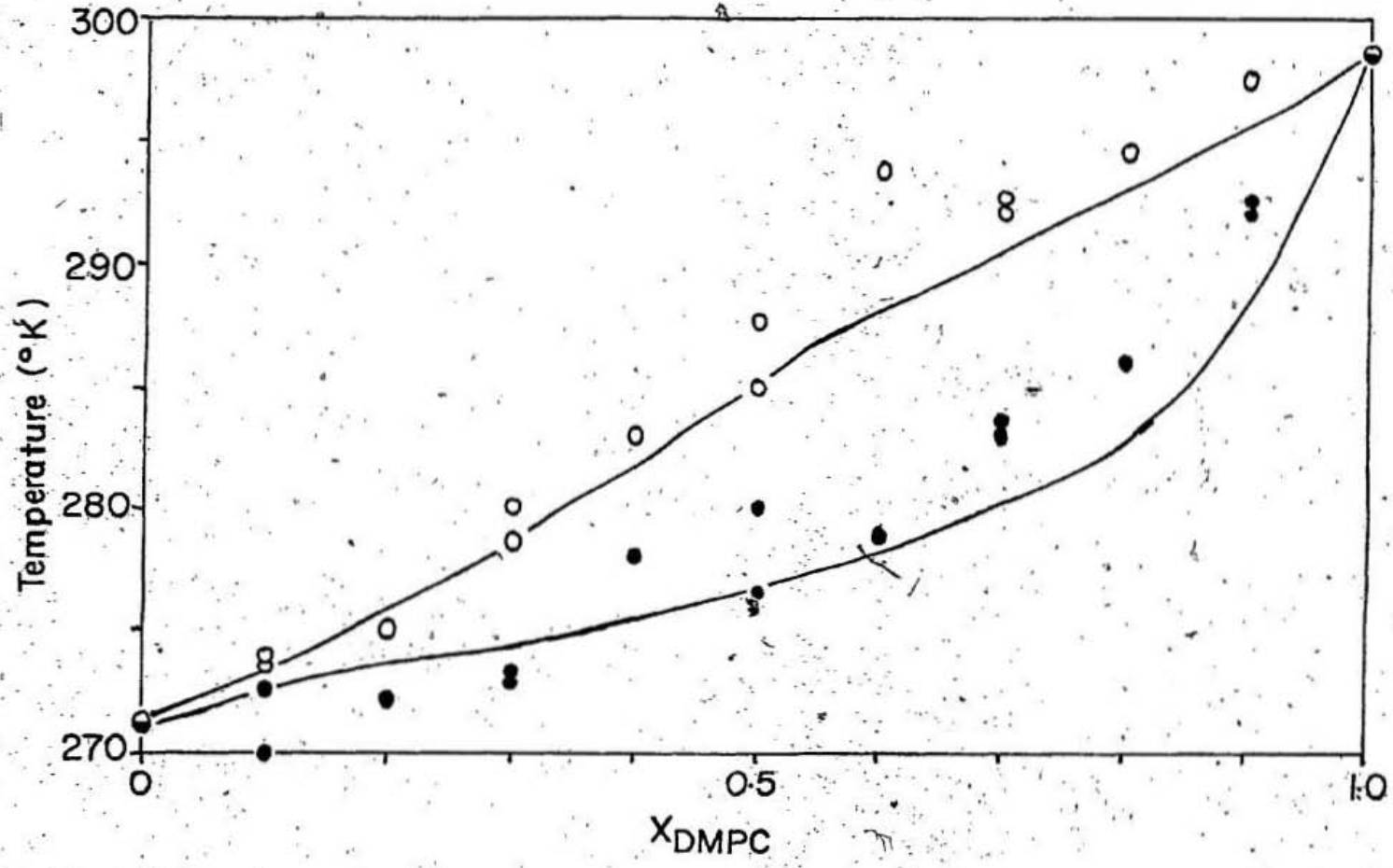

300
290
280
270
Temperature (°K)
0
0·5
1·0
X_{DMPC}

Figure III-33

Phase diagram for mixtures of DMPC with PSPC. (○) liquidus temperatures; (●) solidus temperatures; (——) liquidus and solidus curves computed for ρ_o^{LIQ}=0.70 kcal·mol^{-1} and ρ_o^{SOL} = 0.90 kcal·mol^{-1}.

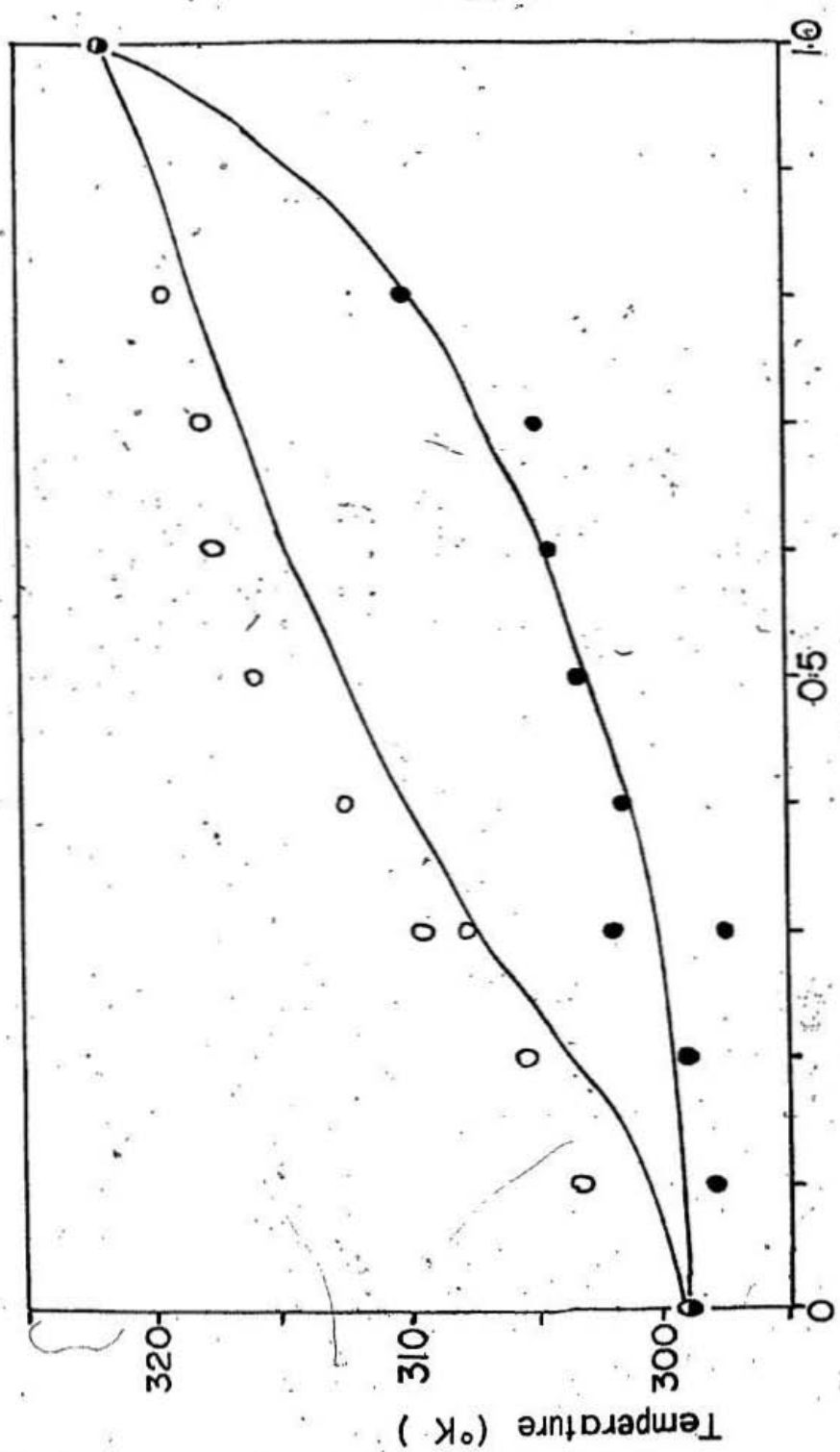
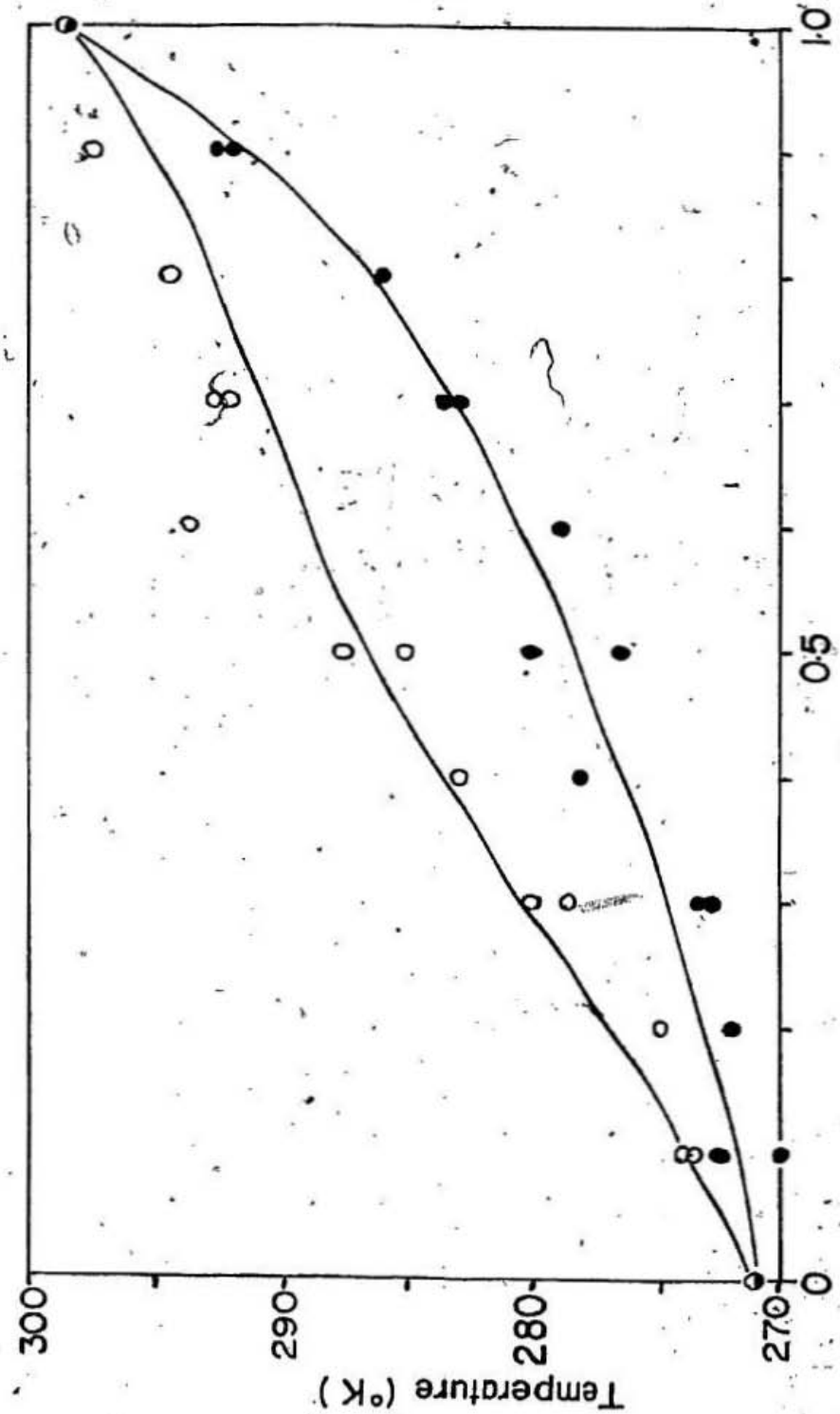

Temperature (°K)
320
310
300
0
0.5
1.0
XPSPC

Figure III-34

Phase diagram for mixtures of DMPC with POPC. (O) liquidus temperatures; (●) solidus temperatures; (——) liquidus and solidus curves computed for $p_o^{LIQ} = 0.23$ kcal·mol^{-1} and $p_o^{SOL} = 0.42$ kcal·mol^{-1}.



discussed above suggest that mixing is more ideal in the liquid-crystal than in the gel for both the DMPC-PSPC and DMPC-POPC mixtures. The ρ_o^{LIQ} values were different for the two mixtures. DMPC mixed more ideally in the liquid-crystal with POPC than with PSPC. This was also true of the gel as suggested by the greater ρ_o^{*SOL} for DMPC-PSPC.

IV DISCUSSION

IV-1 Lecithin Synthesis

A. Single-acid Lecithins

There are now several commercial suppliers of good quality preparations of many single-acid lecithins and there is less need for good synthetic procedures for single-acid lecithins. Nevertheless there are cases where one may have to prepare single-acid lecithins either because of time constraints or because lecithins having polyunsaturated, branched chain or labeled fatty acids are required. The results of the study of various procedures for the synthesis of single-acid lecithins indicated that, of those examined, the procedure of Gupta *et al.* (1977) is the method of choice. The lower temperature required would be expected to be less detrimental to labile fatty acids than the higher temperatures used in the modification of the procedure of Cubero-Robles and van den Berg (1969) employed by Keough and Davis (1979). Also, the lower anhydride:GPC ratio needed and the high yields obtained could result in substantial economy in cases were the fatty acids are expensive or difficult to prepare. The presence of trace amounts of 1,3-diacyl-*sn*-glycero-2-phosphocholine in the lecithin preparations would not cause serious problems in many of the applications of these preparations. The thermotropic properties of the 1,3-lecithins are only slightly different from those

of the 1,2-lecithins (Seelig et al., 1980) and the very small amounts of 1,3-lecithins would not introduce any significant changes in the thermotropic behavior of the 1,2-lecithins.

B. Mixed-acid Lecithins

In the early stage of this work there were no mixed-acid lecithins of reasonable purity available from commercial sources. Now, the variety of commercially available products is still limited. For this reason it was necessary that a procedure for the preparation of high purity positional isomers of mixed-acid lecithins be found. Three different synthetic procedures were investigated.

Keough and Davis (1979) had developed a method for the hydrolysis of saturated single-acid lecithins using phospholipase A_2 followed by acylation of the 2-lysolecithin (1-acyl-sn-glycero-3-phosphocholine) by a modification of the procedure of Cubero-Robles and van den Berg (1969) using saturated fatty acid anhydrides. This procedure had resulted in significant acyl migration (4 to 22%) with moderate yields (10 to 55%). When applied to the preparation of saturated-unsaturated mixed-acid lecithins this procedure was only moderately successful. As shown in Table III-2, the yields ranged from 20 to 42% with the amounts of the unwanted reversed positional isomers caused by acyl group migration varying from 14 to 26%. This relatively

high acyl migration presented some difficulties particularly in the case of the first pair of positional isomers of saturated-unsaturated mixed-acid lecithins studied (SOPC and OSPC). Because of the small differences in the thermotropic properties between these isomers, small effects due to contamination with the wrong isomer could be important. This procedure also required twelve moles of fatty acid to prepare one mole of mixed-acid lecithin (assuming 100% yields) and unsaturated fatty acids are more expensive than saturated fatty acids. Since these procedures could eventually be used to synthesize mixed-acid lecithins containing the more labile polyunsaturated fatty acids, the high temperatures used during the acylation were also a cause for concern.

The attempts to adapt a procedure reported by Warner and Benson (1977) resulted in very poor yields and the products contained varying amounts of the reversed positional isomers (Table III-2). These authors had reported good yields of single-acid lecithins prepared using this procedure. It is possible that the presence of the acyl chain at the sn-1 position of the lysolecithin may make the addition of the second acyl chain at the sn-2 position more difficult. The mild acylation condition and short acylation time might be insufficient to overcome this potential difficulty. There is however an alternate explanation for the poor yields obtained in the mixed-acid lecithin synthesis. The solvent used for the acylation of the lysolecithin was

dimethylsulfoxide (DMSO) and although the solvent was dried before use by distillation from calcium hydride, DMSO is notoriously hydroscopic. The possibility that the low yields were due to the presence of water in the solvent cannot be eliminated.

The most successful procedure for the reacylation of lysolecithin used in the course of this work was the slightly modified method of Gupta *et al.* (1977). The results summarized in Table III-2 show that this procedure gave good yields and consistently low acyl migration. The most consistent syntheses were obtained when this acylation procedure and was used in conjunction with the hydrolysis procedure of Keough and Davis (1979). This hydrolysis was easier and faster than the procedure of Chakrabarti and Khorana (1975). There was also no exposure of the lysolecithin to borate and ethanol that had caused breakdown of some lyso-oleoyl lecithin preparations. As the results shown in Tables III-5 and III-6 suggested, lyso-oleoyl lecithin prepared using the borate buffered solution of phospholipase A_2 described by Chakrabarti and Khorana (1975) can breakdown to GPC when subsequently exposed to ethanol. In light of the failure to reproduce this breakdown using a second batch of ethanol, the possibility that the breakdown was caused by some contaminant in the ethanol cannot be eliminated. Nevertheless, I recommend the combination of the hydrolysis of Keough and Davis (1979) and the acylation procedure of Gupta *et al.* (1977) for the synthesis of

mixed-acid lecithins. These procedures were used in the preparation of AOPC and OAPC.

It should be pointed out that several other procedures for the acylation of lysolecithin have been reported. Roseman et al., (1978) had used acyl anhydrides in the presence of the sodium salts of the fatty acids to prepare mixed-acid lecithins. This procedure and the procedure of Lammers et al., (1978) have been used by Coolbear and Keough (unpublished) to prepare mixed-acid lecithins and only poor yields were obtained using these procedures. Mason et al. (1981b) have synthesized saturated mixed-acid lecithins using the hydrolysis procedure of Keough and Davis (1979) followed by reacylation of the lysolecithin using five mole equivalents of fatty acid anhydride and one mole equivalent of 4-pyrrolidinopyridine in dry chloroform at 35°C. The yields were very high (84 to 93%) and the products contained only small amounts (0.7 to 1.1%) of the reversed positional isomers.

The purification of gram quantities of synthetic mixed-acid lecithins necessitated the use of large chromatographic columns. In the early preparations, the synthetic products were purified using silicic acid columns (Keough and Davis, 1979). Attempts to replace this slow column procedure with a procedure reported by Radin (1978) for the use of prepacked Lobar Columns were not particularly promising. In later preparations, the silicic acid column chromatogra-

phy was replaced by chromatography on CM-52 carboxymethyl cellulose columns (Comfurius and Zwaal, 1977). The elution of the CM-52 columns was much faster than that of the silicic acid columns and the yields from the CM-52 columns were nearly quantitative. By eliminating the earliest fractions (containing most of the 1,3-lecithin) 1,2-lecithin containing only trace amounts of 1,3-lecithin were obtained. It is also noteworthy that, unlike the silicic acid columns which are discarded after use, the CM-52 columns were used for three to four purifications and, thus, they were somewhat more economical.

The use of the acylation procedure of Gupta et al. (1977) also involved an initial purification of the lecithin product on a mixed-bed ion exchange column. Because the water content in the eluant from this ion exchange column was relatively high (10%) the removal of all the water prior to the CM-52 column chromatography was very time consuming. To avoid the need to keep the lecithins on a rotary evaporator for long periods of time, the compositions of the eluants from the ion exchange columns were adjusted to give the upper and lower phases of Folch et al. (1957). The lecithin partitions into the lower phase which has a low water content and can therefore be dried quickly. This step also aids in the removal of water-soluble contaminants.

The introduction of the crystallization step following the precipitation of the lecithins from chloroform with

acetone resulted in preparations having higher purity as indicated by a sharpening of the dsc endotherms. Nevertheless, the possibility that these preparations contain very small amounts of contaminants which were not detected by the tlc, glc and dsc analyses used in this work cannot be completely ruled out.

The estimates of the amounts of reversed position isomers in the mixed-acid lecithins were based on the fatty acid composition of the lysolecithin obtained by phospholipase A_2 digestion of the lecithins. In the positional analyses of many of the mixed-acid lecithins, the composition of the fatty acids released from the sn-2 position in these digestions indicated higher acyl migration than did the lysolecithin composition. It has been reported (Pluckthun and Dennis, 1982) that, during the phospholipase A_2 hydrolysis, some of the fatty acyl groups at the sn-1 position were hydrolyzed by the enzyme. This did not appear to be caused by a lack of specificity of the phospholipase A_2 but was the result of migration from the sn-1 position to the sn-2 position. The fatty acid that had migrated to the sn-2 position was then released by the enzyme reaction with the formation of GPC. If this occurred during the positional analyses used in this work, the composition of the resulting free fatty acids would indicate a lower positional purity than that indicated by the composition of the lysolecithin. For this reason, the positional purity indicated by the composition of the lysolecithin was taken as being more

reliable than the positional purity indicated by the composition of the liberated fatty acids.

Acyl group migration during phospholipase digestion and during reacylation was responsible for the formation of the reversed isomers formed during the syntheses. The migration is catalyzed by base (Pluckthun and Dennis, 1982) and it is noteworthy that the N,N-dimethylaminopyridine used in the acylation of Gupta et al., (1977) is a weaker base than the sodium oxide used in the procedure of Keough and Davis (1979). The stronger base used in the latter procedure may account for the higher acyl migration that occurred in the syntheses using this procedure.

IV-2 The Thermotropic Behavior of Mixed-Acid and Single-Acid Lecithins

A. The Shapes of the Transition Endotherms

The heating endotherms obtained with the three pairs of positional isomers of saturated-unsaturated mixed-acid lecithins (Figure III-2) were asymmetric. The endotherms were slightly skewed to the high-temperature side of the transitions. Asymmetric endotherms have been observed before with saturated mixed-acid lecithins (Keough and Davis, 1979; Stumpel et al., 1981; Chen and Sturtevant, 1981). Chen and Sturtevant (1981) have suggested that the high-temperature asymmetry seen on endotherms obtained with some of their saturated mixed-acid lecithins was due to the presence of

significant amounts of the reversed positional isomers in their preparations. This appears unlikely because of the asymmetry seen in the endotherm for pure OPPC. This lecithin was obtained from Sigma Chemical Company and found to be greater than 99% pure by tlc. Positional analysis of this lecithin showed that less than one percent of POPC was present. Yet the endotherms obtained with this preparation showed a greater asymmetry than the endotherms obtained with SOPC containing 6% of OSPC. It is also noteworthy that the endotherms obtained by Stumpel et al. (1981) with preparations of saturated mixed-acid lecithins containing less than two percent of the reversed positional isomers showed substantial asymmetry. It is possible that trace contaminants, that are not detected by analytical procedures used here, are responsible for the asymmetry seen in these endotherms. The asymmetry observed in the endotherms obtained with the saturated-unsaturated mixed-acid lecithins studied here might be the result of thermal lags, but the fact that Chen and Sturtevant (1981) observed asymmetric endotherms for saturated mixed-acid lecithins using a high sensitivity calorimeter operated at much lower heating rates suggested that this was unlikely.

Both Chen and Sturtevant (1981) and Mason et al. (1981b) have suggested that in mixed-acid lecithin bilayers in the gel phase there may exist distinct regions of non-interdigitated and interdigitated chain packing (the latter region could itself contain interdigitated chains packed in

different conformations (Mason et al., 1981b). The effects of cooling rates on the behavior of these saturated mixed-acid lecithins also suggested that the packing arrangements in one or more of these regions are metastable. Such a situation occurring in the saturated-unsaturated mixed-acid lecithins could cause the high-temperature asymmetry seen in the endotherms for these lecithins.

The gel to liquid-crystalline phase transition in hydrated phospholipid bilayers has been thought to be a simple two-state transition. This assumes that each of the lipids in the bilayers exist in either the gel state or liquid crystalline state. Since the asymmetric endotherms shown in Figure III-2 could result from two overlapping transitions (eg., gel to intermediate state and intermediate to liquid crystal), an attempt was made to check for simple two-state transition behavior. Lumry et al., (1966) have suggested that the equal area test of Hill (1963) can be used for this purpose. Plots of $\int_{T_c}^{T} C_p$ vs T were constructed from phase transition endotherms of indium, hexadecane and a variety of lecithin dispersions. Figure IV-1 shows a typical plot for DPPC. For two-state transitions the areas "ABE" and "CBD" should be equal. The results of the application of this test are shown in Table IV-1. Within the precision and variability involved in our experimental determinations, the area ratios of the mixed-acid lecithins were not different from the ratios obtained for transitions that have been considered to be two-state transitions.

B. Transition Temperatures and Transition Enthalpies

i. Transition Temperatures

The transition temperatures of each pair of positional isomers were intermediate between those of the respective parent single-acid lecithins. However, for each isomeric pair the transition temperatures of both isomers were closer to the transition temperature of DOPC than to the transition temperature of the respective saturated single-acid lecithin. It was suggested by Berde *et al.* (1980), on the basis of a theoretical approach, that the effects of double bonds on molecular shape and molecular packing would be most important for determining transition temperatures in saturated-cis-unsaturated mixed-acid lecithins and the behavior of the transition temperatures observed in this work was consistent with this suggestion. There were differences between the transition temperatures of the positional isomers in each pair of isomers. These differences will be discussed below (Section IV-2C).

ii. Transition Enthalpies

The transition enthalpies of the saturated-unsaturated mixed-acid lecithins were smaller than the enthalpies of either parent single-acid lecithin. If one assumes that an equilibrium exists between gel phase and liquid-crystalline phase then $\Delta G=0$ for temperatures in the range of the phase transition, and at T_{max} the entropy change (ΔS) for the

Figure IV-1

Plot of $\int_{T_c}^{T} C_p$ vs T for DPPC after Lumry et al., 1966.

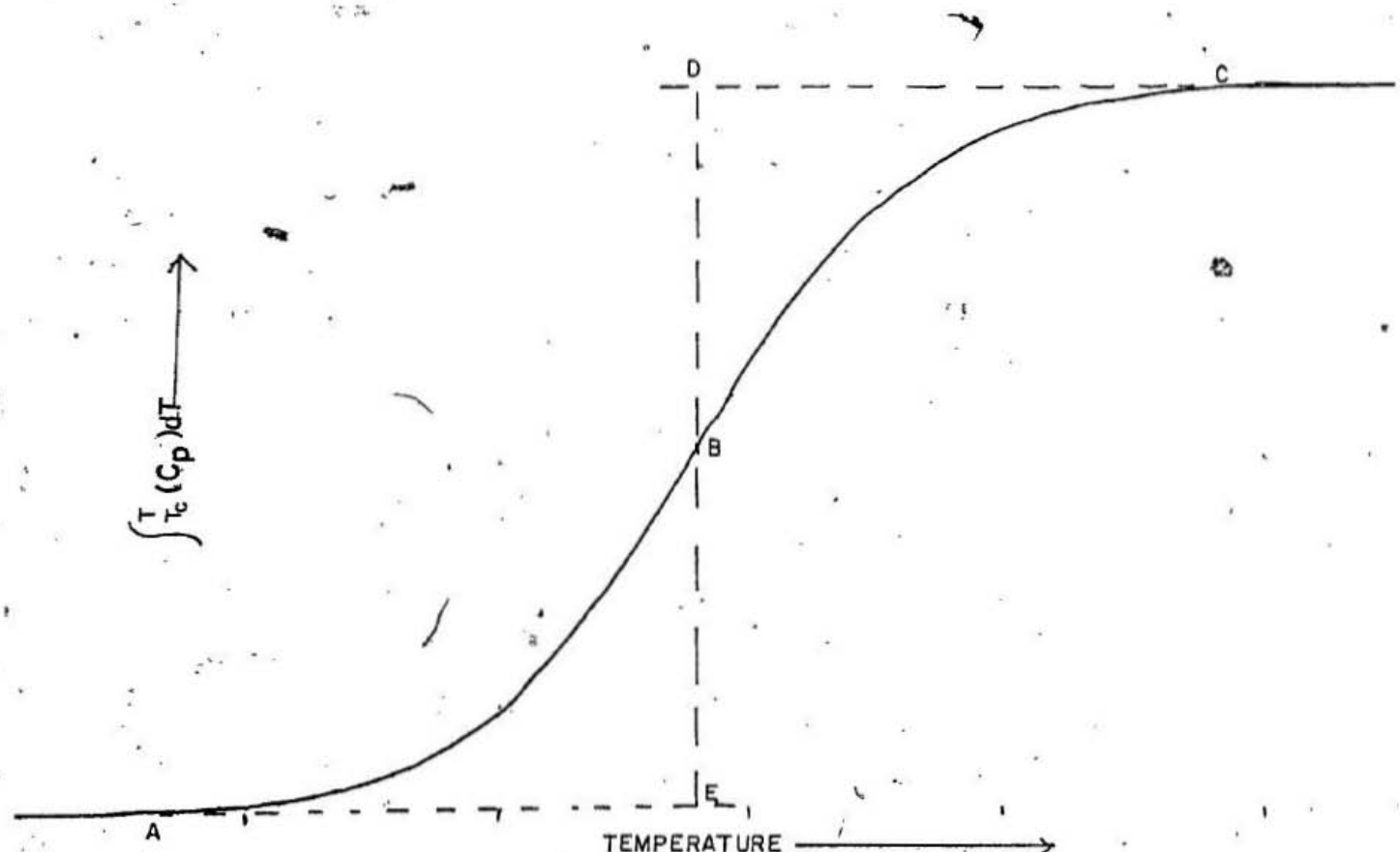

D
C
B
A
E
$\int_{T_c}^{T} (C_p)dT$
TEMPERATURE

TABLE IV-1

HILL TEST

(Excess heat Capacity vs Temperature)

Sample	Area (lower) (kcal·deg^{-2}·mol^{-1})	Area (upper) (kcal·deg^{-2}·mol^{-1})	Area (lower)/ Area (upper)
Indium	1.13	1.29	1.14
Hexadecane	23.5^{+}	17.2^{+}	1.37
DPPC	2.44	2.48	0.98
SOPC^{I}	3.02	3.68	1.22
SOPC^{II}	2.69	3.92	1.46
OSPC^{I}	2.35	1.88	0.80
OSPC^{II}	1.42	1.48	1.04
DSPC	3.34	2.70	0.82
DOPC	1.96	1.06	0.54

$^{+}$units are kcal·deg^{-2}·g^{-1}

Superscripts I and II denote different preparations of these phospholipids.

transition can be evaluated by:

$$\Delta S = \frac{\Delta H}{T_{max}}$$

Table IV-2 shows the ΔH and ΔS values for the gel to liquid-crystal transitions of the lecithins studied here. The difference in the entropies between the gel and the liquid-crystalline states was greater for DOPC, DSPC and DAPC than for the saturated-unsaturated mixed-acid lecithins. It has been shown by Seelig and Seelig (1977) that, with the exception of the carbon atoms near the cis double bond of the oleate chain, the segmental order parameters of POPC and DPPC are nearly the same at the same reduced temperature (T^e) where $T^e = (T - T_c)/T_c$ (Seelig and Browning, 1978). Thus it seems reasonable to assume that the entropy of both the mixed-acid lecithins and the single-acid lecithins in the liquid-crystalline state are nearly the same. If this is the case, then the smaller ΔS values for the mixed-acid lecithins implies that, in the gel state, the mixed-acid lecithins are more disordered than are the saturated single-acid lecithins. This might be due to the difficulties involved in packing kinked unsaturated chain adjacent to a straight saturated chain. Order parameters for the hydrocarbon region of DOPC at temperatures near the phase transition temperature have not been determined and thus it is difficult to relate the ΔS values obtained for the mixed-acid lecithins studied here to the ΔS value obtained for DOPC, at least in terms of the ordering of the

TABLE IV-2

Entropy Changes (ΔS) for the Gel to Liquid-crystalline Phase Transitions

LECITHIN	ΔH (kcal·mol^{-1})	ΔS (e.u.)
OPPC	4.6	17.3
POPC	5.4	19.8
SOPC	6.6	23.5
OSPC	6.4	22.6
AOPC	4.2	14.7
OAPC	6.9	23.7
DOPC	9.3	32.1
DSPC	8.2	27.8
DAPC	14.9	43.9

acyl chains in the gel state.

iii. Transition Widths ($\Delta T_{\frac{1}{2}}$)

The widths at half maximum excess heat capacity of the transition endotherms ($\Delta T_{\frac{1}{2}}$) obtained with the dispersions of the single-acid and mixed-acid lecithins studied here were comparable to those that have been obtained with dispersions of saturated mixed-acid lecithins using low sensitivity calorimeters (Keough and Davis, 1979; Stumpel et al., 1981). Although the half height widths have been used as measure of the cooperativity of the phase transition, this must be done with caution since ($\Delta T_{\frac{1}{2}}$) values are affected by the operating conditions of the calorimeter. Since the endotherms reported in this work were all obtained under identical operating conditions, comparison of $\Delta T_{\frac{1}{2}}$ values reported here is valid.

C. Differences Between Positional Isomers

There are two structural differences between fatty acid positional isomers of saturated-unsaturated mixed-acid lecithins that could contribute to differences in their thermotropic behavior. One of these is a difference in the depth in the bilayer of the cis double bond between the 1-saturated-2-unsaturated isomer and the 1-unsaturated-2-saturated isomer. X-ray studies on lecithin crystals (Pearson and Pascher, 1979) and deuterium nmr, neutron diffrac-

tion and Laser Raman studies on lecithin dispersions (Seelig and Seelig, 1975; Haberkorn *et al.*, 1977; Buldt *et al.*, 1978; Gaber *et al.*, 1978; Oldfield *et al.*, 1978; Seelig and Waespe-Sarcevic, 1978; Zaccai *et al.*, 1979) have shown that the sn-1 and sn-2 chains of lecithins are not equivalent in crystals or bilayers. While the sn-1 chain is fully extended, the initial segment of the sn-2 chain near the glycerol is oriented parallel to the bilayer surface and the remainder of the sn-2 chain is oriented parallel to the sn-1 chain (see Figure I-1). In hydrated bilayers of both single-acid and mixed-acid lecithins the carbon atoms of the sn-1 chain are located about 1.8 Å (or one and a half carbon-carbon bond lengths) deeper in the bilayer than corresponding carbon atoms of the sn-2 chain (Buldt *et al.*, 1978). Thus in OSPC the double bond is 1.8 Å deeper in the bilayer than is the double bond in SOPC. It was reported by Barton and Gunstone (1975) that the transition temperatures and enthalpies of dioctadec-*cis*-enoyl lecithins and 1-octadecanoyl-2-octadec-*cis*-enoyl lecithins were influenced by the position of the double bonds in the acyl chain. For the dioctadec-*cis*-enoyl PC a minimum in the transition temperatures was observed when the double bond was located at C-9 to C-11 and for the 1-octadecanoyl-2-octadec-*cis*-enoyl PC a minimum occurred when the double bond was at C-9. The change in the depth of the double bond resulting from changing the position of the oleate chain from the sn-2 to the sn-1 position might account for the small increase in tran-

sition temperature of OSPC over that of SOPC. A change in the depth of the double bond of this magnitude, however, would not be expected to account for the difference in the transition temperatures between AOPC and OAPC. Moreover, if the change in the depth of the double bond was the only feature responsible for the differences in the transition temperatures between the positional isomers, the findings of Barton and Gunstone (1975) would predict that OPPC should have a higher transition temperature than POPC. This is clearly not the case. On the basis of the difference in the depth of the double bond the transition temperature of OPPC would be expected to be 2.3°C higher than that of POPC. The transition temperature of OPPC was about 6.7°C lower than that of POPC. It appears that changing the position of the long (oleate) and short (palmitate) chains on the glycerol moiety must override any effects due to changes in the depth of the double bond.

It has been suggested that the differences in the lengths of the sn-1 and sn-2 chains of saturated mixed-acid lecithins could be used to account for the differences in transition temperature. (Keough and Davis, 1979; Stumpel et al., 1981; Chen and Sturtevant, 1981; Mason et al, 1981b). The saturated-unsaturated mixed-acid lecithins studied here also have intramolecular differences in chain length. For the lecithins containing oleate and either palmitate or arachidate, the two acyl chains are different by about two methylene units plus small effects due to the fact that the

cis double bond is shorter than a single bond. There is also a difference in the chain lengths between oleate and stearate arising from the introduction of the cis double bond although this difference is small. Seelig and Waespe-Sarcevic (1978) have shown that the cis-double bond in the liquid-crystalline bilayers of POPC is oriented almost parallel to the bilayer normal. This orientation can be obtained by twisting the x-1 or x+1 bond or both (where the x bond is the C_{9-10} double bond) by 30° and introducing a gauche rotamer in the x-2 or x+2 bond or both. If only one twist plus gauche rotamer is used, the oleate chain contains two linear segments parallel to the bilayer normal. Each segment of the oleate chain would have a slightly different axes, and the oleate chains would penetrate the bilayer by 0.3Å less than would a stearate chain in the same position on the glycerol. The use of two twists plus two gauche rotamers results in a oleate chain in which the two linear segments are on the same axis but the axis of the double bond would be shifted laterally by a small amount. This latter arrangement would increase the difference in the penetration between oleate and stearate to 0.6 Å.

In each of the three pairs of isomers of saturated-unsaturated lecithins, the 1-short-2-long isomer had the higher transition temperature of the pair. This pattern is consistent with the observations on the saturated mixed-acid lecithins in which the 1-short-2-long isomers had higher transition temperatures than the corresponding 1-long-2-

short isomers (Keough and Davis, 1979; Stumpel et al., 1981; Chen and Sturtevant, 1981; Mason et al., 1981b). The pattern is broken only by the pair of isomers having decanoate and stearate chains (Mason et al., 1981b) and this is likely due to some fundamental difference in packing (for example extensive chain interdigitation) which occurs when there is so great a difference in the chain lengths. Such interdigitation has been proposed to account for the anomalous transition temperatures of synthetic sphingomyelins where there is also a large chain length difference (Barenholz et al., 1976).

The higher transition temperatures that had been observed for the 1-short-2-long isomers of the saturated mixed-acid lecithins, where there were chain differences of up to four carbon atoms, could be explained by assuming that the acyl chains of opposing lecithins do not interdigitate. The longer chain may be foreshortened (perhaps by the introduction of gauche rotamers) (Keough and Davis, 1979; Stumpel et al., 1981; Mason et al., 1981b) so that the bilayer thickness of the 1-long-2-short isomers would then be less than that for the 1-short-2-long isomer. The decreased bilayer thickness would result in reduced van der Waals interactions between acyl chain, and the foreshortening of the long chain would probably cause increased disorder in the gel. Both of these features would lead to lower transition temperatures for the 1-long-2-short isomer. The behavior of the saturated-unsaturated mixed-acid lecithins

could also be accommodated by this approach.

D. Biological Implications

The transition temperatures of the 1-saturated-2-unsaturated lecithins are different from those of the 1-unsaturated-2-saturated lecithins, in some cases by as much as 7-8°C, and thus membranes containing different positional isomers may also have different physical properties. It is possible, therefore, that the predominance of the 1-saturated-2-unsaturated isomers in biological systems is not the result of historical accident. It is noteworthy that the 1-unsaturated-2-saturated isomers do occur in some biological systems (Hildebrand and Law, 1964; Dyatlovitskaya et al., 1974; Rottem and Markowitz, 1979) and these systems have some other characteristics that are different from other biological systems containing the more common positional isomers.

IV-3 Cholesterol-Lecithin Interactions

A. Differences Between Lecithins

Recent studies of the effects of cholesterol on the thermotropic behavior of aqueous dispersions of saturated single-acid lecithins have provided considerable insight into the possible arrangements of lecithin-cholesterol bilayers. In addition to the decrease in the enthalpy of the gel to liquid-crystal phase transition caused by the

addition of cholesterol to DPPC (Ladbrooke et al., 1968), it has been shown that the effects of low concentrations of cholesterol (≤ 20mol%) on the phase transition behavior of DPPC involved the coexistence of two phases at least in the gel phase (Estep et al., 1978; Mabrey et al., 1978; Lentz et al., 1980; Melchoir et al., 1980). Transition endotherms obtained by differential scanning calorimetry of aqueous dispersions of DPPC containing up to 20mol% cholesterol appeared to have two components, a sharp endotherm superimposed on a broad endotherm (Estep et al., 1978; Mabrey et al, 1978). Two-component endotherms have also been observed with dispersions of sphingomyelin-cholesterol mixtures (Estep et al., 1979) and possibly with dispersions of mixtures of saturated single-acid phosphatidylethanolamines with cholesterol (Blume, 1980). It has been suggested that the sharp component of the two-component endotherms was the result of a gel to liquid-crystal transition occurring in a domain of nearly pure lipid (Estep et al., 1978, 1979; Mabrey et al., 1978; Snyder and Freire, 1980). The broad component may arise either from transitions in a cholesterol-enriched domain (Mabrey et al, 1978) or from transitions occurring in the interfacial region between nearly pure lipid domains and cholesterol-rich domains (Estep et al., 1978; Snyder and Freire, 1980).

Lentz et al., (1980) have measured the microviscosity DPPC-cholesterol dispersions by fluorescence polarization using the probe diphenyl hexatriene (DPH). These authors

have shown that plots of the "microviscosity activation energy" [$\Delta H^{\neq} = R\ \partial \ln \eta / \partial \ln (T^{-1})$]; Lentz et al., 1978) against temperature resulted in the superposition of two peaks, a sharp peak similar to that observed with pure DPPC and a broad peak. They have suggested the existence of a phase boundary in DPPC-cholesterol mixtures at $X_{chol} = 0.20$.

The addition of cholesterol to saturated-unsaturated mixed-acid lecithins studied here and to their corresponding "parent" single-acid lecithins resulted in a decrease in the maximum excess heat capacity and in the enthalpies of the thermotropic transitions. In this way these lecithins behave like the saturated single-acid lecithins and the sphingomyelins that have been studied previously. The endotherms observed here with both the mixed-acid and the single-acid lecithins containing cholesterol could be resolved into two components similar to those that have been reported by Estep et al. (1978, 1979) and Mabrey et al. (1978). Endotherms from the DSPC-cholesterol mixtures and the OAPC-cholesterol mixtures showed little, if any, asymmetry and could be considered as having only one component. Nevertheless, these endotherms might arise from a superposition of two components with equal or nearly equal T_{max}.

There were differences in the effects of cholesterol on the behavior of the phase transition endotherms between all the lecithins studied here. The results obtained in these studies were also different from those that have been

obtained with other lipid-cholesterol mixtures (Estep et al., 1978, 1979; Mabrey et al., 1978; Melchoir et al., 1980; Blume, 1980). Differences were observed in the effect of cholesterol concentration on the total transition enthalpies, on the relative contributions of the components of the endotherms to the total endotherms and on the T_{max} of the components of the endotherms.

Addition of as little as 30mol% cholesterol to OSPC resulted in no detectable transition while a phase transition was detected in DAPC and DOPC containing equimolar amounts of cholesterol. No transitions were detectable in SOPC and AOPC containing 40mol% cholesterol, but DSPC and OAPC containing 40mol% cholesterol did exhibit transitions. For DSPC and OAPC, transitions could not be resolved from base line at X_{chol}=0.48 and 0.50 respectively. It has been shown by Mabrey et al. (1978) that transitions are detectable using high sensitivity dsc in DPPC-cholesterol dispersions containing up to 50mol% cholesterol. The transitions of palmitoylsphingomyelin have also been shown to disappear at about 50mol% cholesterol (Calhoun and Shipley, 1979).

There were also differences in the effects of cholesterol on the enthalpies of the two components of the endotherms for the lecithins studied here and between these and lipid-cholesterol mixtures studied by others. Addition of 17mol% cholesterol to OSPC eliminated the narrow components of the endotherms while the endotherms of the other

three mixed-acid lecithins (SOPC, AOPC and OAPC) exhibited narrow components at 17mol% but not at 23mol% cholesterol. In the case of the "parent" single-acid lecithins the narrow components of the endotherms were observed up to 30, 40 and 50mol% cholesterol for DSPC, DOPC and DAPC respectively. In all the lecithin-cholesterol mixtures there were progressive decreases in the enthalpies attributable to the narrow components with increasing concentrations of cholesterol. For the three "parent" single-acid lecithins and for the two mixed-acid lecithins, AOPC and OAPC, there was a nearly linear relationship between the cholesterol concentration and the enthalpies of the narrow components. This relationship may also be linear for the SOPC-cholesterol mixtures, but the decrease in the enthalpies of the narrow component for the OSPC-cholesterol mixtures was distinctly non-linear and was like that of palmitoylsphingomyelin-cholesterol mixtures (Estep et al., 1979).

At any given concentration of cholesterol the enthalpy of the narrow component in OSPC-cholesterol mixtures was less than that of SOPC-cholesterol mixtures. A similar pattern was observed for the enthalpies of the narrow components obtained for mixtures of cholesterol with AOPC and OAPC. The enthalpies associated with the narrow components of AOPC-cholesterol were lower than those in OAPC-cholesterol mixtures containing the same concentration of cholesterol.

Thus it appears that the intramolecular differences in acyl chain length may also exert an influence on the effect of cholesterol on the enthalpies associated with the narrow components of the endotherm of mixed-acid lecithin-cholesterol mixtures that overrides any influence of the positional distributions of the saturated and unsaturated chains.

The enthalpies associated with the broad components of the endotherms increased with increasing cholesterol for all the mixed-acid lecithin-cholesterol mixtures. The enthalpies of the broad components reached a maximum at 17mol% cholesterol in OSPC and SOPC, and at 23 to 30mol% cholesterol in AOPC and OAPC. Further addition of cholesterol led to a decrease in the enthalpies of the broad components. No maxima were observed for DOPC-cholesterol and DAPC-cholesterol mixtures but a broad maximum (from 17 to 30% cholesterol) was observed for the DSPC-cholesterol mixtures.

Any attempt to interpret the differences in the effects of cholesterol on the behavior of the lecithins studied here requires that one make some assumptions about the origins of the narrow and broad components of the endotherms. There seems to be general agreement that the narrow components reflect transitions occurring in nearly pure lipid (Estep et al., 1978, 1979; Mabrey et al., 1978; Lentz et al., 1980; Snyder and Freire, 1980; Slater and Caille, 1981). The

broad component, on the other hand, has been attributed to a transition occurring either in a cholesterol-rich domain or in an interface region between cholesterol-rich and pure lipid domains. Estep et al., (1978) have pointed out that, if the broad component arises from a cholesterol-rich domain, there must exist a boundary region that does not make any significant contribution to the observed endotherms. These authors have also shown that the effects of changes in pressure on the two components of the endotherms on DPPC-cholesterol mixtures were very similar. They argue that this observation suggests that these regions giving rise to the two components are not distinct phases and since a cholesterol-rich phase would be expected to differ substantially from the pure lipid, it was more likely that the broad component arises from the interfacial region. The lipid in interfacial region would be essentially pure lipid located adjacent to lipid-cholesterol and would be very similar to the bulk lipid in the pure lipid domain, thus able to react to changes in pressure in the same way as pure lipid.

Whatever the source of the broad components, the results obtained in this work indicated that there were quantitative, if not qualitative, differences in the way in which cholesterol interacts with different lecithins in the gel state and that the interaction of cholesterol was influenced by the structure of the lecithins. The effects of cholesterol concentration on the enthalpies of the narrow

components of OSPC were different from those seen with SOPC. Assuming that the enthalpies of the narrow components reflect the size of the pure lipid domains, the results indicated that for a fixed concentration of cholesterol, there was less OSPC in the pure lecithin domains of OSPC-cholesterol mixtures than there was SOPC in the pure lecithin domains of SOPC-cholesterol mixtures. This may be due to a greater proportion of OSPC being located in the cholesterol-rich domain than SOPC or, as discussed by Estep et al. (1978), this may indicate that there are larger interfacial regions in OSPC-cholesterol mixtures than in SOPC-cholesterol mixtures. This would imply that the OSPC-cholesterol system was more ramified than the SOPC-cholesterol system. This would be the case if cholesterol mixing was more ideal with OSPC than with SOPC.

Preferential interactions of cholesterol with different lipids have been reported before (de Kruijff et al., 1973; van Dijck et al., 1976) and these preferential interactions were influenced by the transition temperature and the head group of the lipids. Nevertheless, the small difference in the transition temperatures between SOPC and OSPC would be unlikely to result in the different effects of cholesterol on the thermotropic behavior of these two position isomers. The greater association of OSPC with cholesterol compared with that of SOPC must be due to the difference between the structures of these isomers that results from the different positional distribution of the acyl chains.

B. Possible Influence of Lipid Structure on Cholesterol Interactions

It has been noted above (Section IV-2C) that there are two structural differences between bilayers of OSPC and SOPC. One of the differences is the depth of the double bond in the hydrocarbon region of the bilayers. In OSPC the cis double bond is located about 1.8 Å deeper in the bilayer than in SOPC. This difference in double bond location could account for a more facile interaction of cholesterol with OSPC than with SOPC. Huang (1977) has pointed out that the cholesterol molecule has a flat α -face and a β -face that was puckered because of the methyl groups at C-10 and C-13 of the sterol ring. He has suggested that the non-linear unsaturated acyl chain can accommodate the puckered β -face of the cholesterol with less perturbation than would be caused if the β -face were adjacent to the straight saturated acyl chain. Measurements made on space-filling models indicated that the puckered surface of cholesterol can be better accommodated if the first linear segment of the unsaturated acyl chain extends at least 10 Å into the bilayer. For an oleate chain located at the sn-1 position (as in OSPC), the first linear segment can extend into the bilayer to a depth of approximately 7.5 Å if the twist plus gauche rotamer (see Section IV-2) is introduced on the carbonyl side of the double bond and to a depth of approximately 10 Å if the twist plus gauche rotamer is on the methyl side of the double bond. Huang (1977) has also

suggested that the following coupled isomerization may occur

$$tt\Delta_{9,10}tg \quad [A]$$

$$gt\Delta_{9,10}tg \quad [B] \qquad gt\Delta_{9,10}tt \quad [C]$$

and that the presence of cholesterol shifts the equilibrium to arrangement [A]. Because of the "bent" chain conformation of the sn-2 chain near the ester bond the length of the initial linear segment of oleate located at the sn-2 position (as in SOPC) would extend into the bilayer to a depth of 5.7 Å if the twist plus gauche rotamer was on the carbonyl side of the double bond [C] and to a depth of 8.2 Å if the twist plus gauche rotamer was on the methyl group side of the double bond [A]. If the β-face of cholesterol does interact with the unsaturated acyl chain as suggested by Huang (1977) then the measurements discussed above would be consistent with there being a smaller probability for van der Waals interactions between SOPC and cholesterol than for interactions between OSPC and cholesterol. Thus OSPC-cholesterol interactions would be energetically more favorable than SOPC-cholesterol interactions. The P parameter, defined by Snyder and Freire (1980) as a Boltzman exponent proportional to the energy of mixing between lipid-lipid and lipid-cholesterol, would be smaller for OSPC-cholesterol mixing than for SOPC-cholesterol mixing, and the OSPC-cholesterol mixing would be more ideal. This is consistent

with the results obtained in this study. The effects of cholesterol concentration on the enthalpies of the narrow components of the endotherms for OSPC-cholesterol were like those for palmitoylsphingomyelin-cholesterol and for SOPC-cholesterol the effects were more like those of DPPC-cholesterol (Estep et al., 1979).

The model proposed by Huang (1977) involves the formation of hydrogen bonds between the β -hydroxyl group of cholesterol and the sn-1 carbonyl of the lecithin, but there is now some doubt about the presence of hydrogen bonds between cholesterol and the polar region of phospholipids. The above argument, however, does not depend on the formation of hydrogen bonds, but it does require that the cholesterol be oriented in the bilayer approximately as described by Huang (1977). Neutron diffraction studies indicate that cholesterol in lecithin bilayers is, in fact, oriented in this way (Franks, 1976; Worcester and Franks, 1976).

The second major structural difference between the fatty acid positional isomers that might influence their interaction with cholesterol is the extent to which the acyl chains penetrate the bilayer. It has been suggested by McIntosh (1978) that the length of the acyl chains of saturated single-acid lecithins may influence the interaction between cholesterol and these lecithins in the gel phase. It has also been shown that the effects of

cholesterol on DPPC were qualitatively, and possibly quantitatively, different from the effects of cholesterol on DMPC (Mabrey et al. , 1978).

There is a difference in the effective penetrations of the acyl chain at the sn-2 position between the positional isomers, SOPC and OSPC. The slightly greater penetration of the stearate chain at the sn-2 position of OSPC over that of the oleate chain of SOPC was suggested as a potential cause of the difference between the transition temperatures of these isomers. Nevertheless, the difference between the lengths of stearate and oleate is very small and might not be expected to be the only factor responsible for the greater effects of cholesterol on OSPC relative to the effects on SOPC. Thus the depth of the double bond may be the major feature causing the difference in the effect of cholesterol on the thermotropic behavior between SOPC and OSPC.

The results obtained with mixtures of cholesterol with AOPC and with OAPC suggested that any more facile interaction of cholesterol with isomers having the unsaturated oleate chain at the sn-1 position were overridden by differences in the positions of the long and short chains on the glycerol. For the AOPC-OAPC pair, there were more molecules of AOPC associated with a fixed amount of cholesterol (as indicated by the relative contributions of the narrow components of the endotherms to the total enthalpies) than the

number of OAPC molecules associated with the same amount of cholesterol. It is of interest, in this regard, that the plots of the enthalpies, associated with the narrow endothermic components of the endotherms against the concentration of cholesterol were curvelinear for the mixtures of cholesterol with OSPC and possibly SOPC, but were nearly linear for mixtures with AOPC and OAPC. It would appear that the major factor responsible for determining the amounts of each lecithin associated with cholesterol in SOPC-cholesterol and OSPC-cholesterol mixtures was different from that applying to the AOPC-cholesterol and OAPC-cholesterol mixtures.

The endotherms obtained on heating mixtures of cholesterol with the single-acid lecithins DOPC, DSPC and DAPC were different from those obtained with mixed-acid lecithins and were also different from those reported for other single-acid lecithin-cholesterol mixtures (Estep et al., 1978; Mabrey et al., 1978). The behavior of DOPC-cholesterol and DAPC-cholesterol mixtures are unique compared with the behavior of other lecithin-cholesterol mixtures in that these mixtures presented dsc endotherms having a low-temperature asymmetry. A similar low-temperature asymmetry has been observed before in endotherms obtained with mixtures of cholesterol with lignoceroylsphingomyelin and possibly some saturated single-acid phosphatidylethanolamines (Estep et al 1978; Blume, 1980).

With regard to the behavior of the mixtures of cholesterol with the saturated single-acid lecithins, the asymmetry of the endotherms was different for each of the lecithins studied. The endotherms obtained for mixtures of cholesterol with DMPC had a more pronounced high-temperature asymmetry than DPPC-cholesterol mixtures at any given concentration of cholesterol (Mabrey et al., 1978). For the mixtures of cholesterol with DSPC studied in this work, the endotherms had almost no asymmetry. The endotherms obtained for mixtures of cholesterol with DAPC exhibited a definite low-temperature asymmetry. It is interesting to speculate that the effect of cholesterol on bilayers of lecithins having acyl chains with less than eighteen carbons, may be different from the effect on lecithins having acyl chains with more than eighteen carbons. This was suggested by McIntosh (1978) on the basis of X-ray diffraction studies of mixtures of cholesterol with saturated single-acid lecithins. For the saturated single-acid lecithins containing 12 to 16 carbon atoms per acyl chain, addition of cholesterol caused an increase in the bilayer thickness in both the gel and the liquid-crystal. Addition of cholesterol to DSPC resulted in a decrease in bilayer thickness in the gel. In bilayers of lecithins having acyl chains with less than eighteen carbons the cholesterol spans, or almost spans, one half of the bilayer but in DSPC-cholesterol bilayers the chains extend beyond the cholesterol. This may account for the different effect of cholesterol on DSPC bilayers and might also

account for the asymmetry observed in the endotherms for the OAPC-cholesterol mixtures. The acyl chains of OAPC would extend beyond the cholesterol molecule as do the chains of DSPC. It must be pointed out, however, that this is also true of the acyl chains in AOPC but the potential foreshortening of the long chain at the sn-1 position of AOPC may result in AOPC behaving differently from OAPC and DSPC in this respect.

It would appear from these studies and those of others (Estep et al., 1978, 1979, 1981; Mabrey et al., 1978; Snyder and Freire, 1980) that interactions occurring in the hydrocarbon region of the bilayer are important in determining the nature of the lipid-cholesterol interaction. There have been a number of lecithin-cholesterol "complexes" discussed in the literature (Engleman and Rothman, 1972; Martin and Yeagle, 1978; Cornell et al., 1979; Pink and Chapman, 1979; Snyder and Freire, 1980). In this discussion specific lecithin-cholesterol complexes having a significant lifetime and any particular stoichiometry have not been considered. Interactions between the lipids and cholesterol, either short-lived or long-lived, have been discussed without reference to any potential complex formation. Although the life-times of lecithin-cholesterol interactions in the gel will likely be greater than in the liquid-crystal, they do not necessarily imply "complex" formation. It should also be noted that the differences between the cholesterol interactions observed in this work reflect the

interactions occurring in the gel. Although extrapolation of the observed differences to the liquid-crystal cannot be made, unequivocally, there is no apparent reason why some similar differences should not exist in the liquid-crystal. Such differences would, however, probably be more difficult to measure in the liquid-crystal.

C. Biological Implications

The biological implications of the differences in the interaction of cholesterol with different positional isomers of mixed-acid lecithins may be important, especially in light of reports of the occurrence of the more unusual 1-unsaturated-2-saturated phospholipids in some biological systems. Rottem and Markowitz (1979) have observed that a series of Mycoplasmas requiring cholesterol for growth were able to incorporate greater amounts of cholesterol into their membranes than closely related Acholeplasmas. The major phospholipids, phosphatidylglycerols, in the membranes of these Mycoplasmas had predominantly the 1-unsaturated-2-saturated isomers while the membrane lipids of Acholeplasmas had the more common 1-saturated-2-unsaturated distribution (McElhaney and Tourtellot, 1970).

Substantial amounts of 1-unsaturated-2-saturated and 1,2-diunsaturated lecithins have also been found in rat hepatomas (Dyatlovitskaya et al., 1975). The cholesterol concentration in the membrane of rat hepatomas has been shown to be greater than that of normal liver membranes (van

Hoeven and Emmelot, 1972). Dyatlovitskaya et al. (1974) have also reported that sonicated liposomes formed from hepatoma lecithins incorporated substantially less cholesterol than liposomes formed from normal liver lecithins. They suggest that the higher cholesterol in the hepatomas must be mostly endogeneous because the hepatomas have a decreased ability to take up cholesterol from the blood. The incorporation of endogenous cholesterol may be different from the incorporation of exogenous cholesterol and this may account for the conflicting results of higher cholesterol levels in hepatomas and a decreased uptake of cholesterol by sonicated hepatoma lipids.

Thus it appears that the differences in the interaction of cholesterol with different lecithin species may be of biological importance. The relationships, if any, between the types of cholesterol-lipid interactions and membrane structure and function remain to be determined, but the results obtained in this study indicate that cholesterol-lipid interactions are influenced by the structure of lipids.

IV-4 Binary Lecithin Mixtures

Biological membranes contain a variety of different lipid species and the interactions that occur between the different lipids in membranes may have significant effects on structure and function. Because of the complex mixtures that occur in biological membranes, it is difficult to

obtain specific information on lipid-lipid interactions from studies of whole membranes or of the total lipid extracts from the membranes. One approach that can provide information about such interactions is the study of simple model systems composed of binary or ternary mixtures of pure synthetic lipids. The phospholipids that have been studied in such model mixtures previously have been almost exclusively single-acid phosphatidylcholines and phosphatidylethanolamines. The binary mixtures that have been studied in this work are more representative of the lipids found in biological membranes. The mixing of lecithins having saturated acyl chains located at the sn-1 position and unsaturated chains at the sn-2 positions with two saturated single-acid lecithins were studied using differential scanning calorimetry. The objective of these studies was to investigate the effects of small changes in the structures of the hydrocarbon chains of the lecithins on their mixing behavior in bilayers. The phase diagrams that were constructed from the calorimetric data for the mixtures were analyzed assuming that the binary mixtures can be treated as "regular solutions" (Hildebrand and Scott, 1964). The "regular solution" model applies to binary mixtures in which the two types of molecules adopt similar packing arrangements and occupy similar volumes. The lecithin used in the mixtures studied here could be expected to satisfy these two requirements. The approach used to analyze the phase diagrams of the mixtures studied in this work has been described by Lee

(1977). Lee makes an additional assumption that there is no change in the heat capacity (C_p) over the range of the phase transition. High sensitivity calorimetry has been used recently to determine the heat capacity of dispersions of single-acid phosphatidylcholines and phosphatidylethanolamines above and below the transition temperatures of the lipids (Wilkinson and Nagle, 1982). At temperatures below T_{max} the heat capacity increased rapidly with increasing temperatures while above T_{max} there was a much smaller variation of C_p with changing temperature. Nevertheless the C_p values obtained at temperatures 20 degrees above and below T_{max} were almost identical. The measured ΔC_p values at all temperatures were 0.3 to 0.4 $kcal \cdot mol^{-1} \cdot deg^{-1}$ relative to the measured excess heat capacities of the gel to liquid-crystal transition (2.0 to 6.0 $kcal \cdot mol^{-1} \cdot deg^{-1}$ for the pure lecithins).

If mixing of the components in a binary mixture were ideal, the phase diagram that would be expected can be calculated using the following equations (Lee, 1977):

$$X_A^{LIQ} = \frac{\exp(A)(1-\exp(B))}{\exp(A)-\exp(B)} \qquad [IV-2]$$

$$X_A^{SOL} = \frac{1-\exp(B)}{\exp(A)-\exp(B)} \qquad [IV-3]$$

where

$$A = \frac{\Delta H_A}{R}\left(\frac{1}{T_A}-\frac{1}{T}\right)$$

$$B = \frac{\Delta H_B}{R}\left(\frac{1}{T_B}-\frac{1}{T}\right)$$

and ΔH_A and ΔH_B are the enthalpies of the pure components and T_A and T_B are the transition temperatures of the pure components. Evaluation of X_A^{LIQ} and X_A^{SOL} for a number of temperatures between the transition temperatures of the pure components provided the phase diagram that describes ideal mixing of the two components.

For non-ideal mixing, the treatment becomes more complex. The deviation from ideal mixing is defined in terms of the difference in the interaction energies between like and unlike pairs of molecules. Lee (1977) has defined the excess interaction energy that is required to form an unlike pair from two like pairs as ρ_o where

$$\rho_o = Z(2U_{AB} - U_{AA} - U_{BB}) \qquad [IV-4]$$

where Z is the coordination number, U_{AB} is the interaction energy of an unlike pair and U_{AA} and U_{BB} are the like pair interaction energies. By assuming that the entropy of mixing is that of an ideal solution, the excess interaction energy can be attributed to an excess enthalpy of mixing. The phase diagrams for such a mixture can be described by Equations II-1 and II-2. Fitting these equations to the phase diagrams can then provide estimates of the values of ρ_o^{LIQ} and ρ_o^{SOL}.

A. Mixtures of DPPC with POPC and SOPC

The behavior of the mixtures of POPC with DPPC was very similar to that of the SOPC-DPPC mixtures. The shapes of

the endotherms obtained with POPC-DPPC mixtures at any given cholesterol concentration were like those obtained with corresponding SOPC-DPPC mixtures (Figure III-19). These endotherms were very broad and were similar to those obtained for mixtures of DMPC and DSPC (Mabrey and Sturtevant, 1976). The phase diagrams constructed for these mixtures (Figures III-20 and III-21) were also very similar to the phase diagram for DMPC-DSPC mixtures (Shimshick and McConnell, 1973; Mabrey and Sturtevant, 1976). The shapes of the solidus curves suggested immiscibility in the gel state in POPC-DPPC mixtures containing up to 75mol% DPPC (Figure III-20) and in SOPC-DPPC mixtures containing up to 50mol% DPPC (Figure III-21).

The solidus and liquidus curves computed for ideal mixing in the gel and the liquid-crystal are shown in Figures III-20 and III-21. In the POPC-DPPC phase diagram the observed liquidus data showed a good agreement with the ideal liquidus curve. The solidus data deviated dramatically from the ideal solidus curve indicating that these two lecithins did not mix well in the gel - a finding consistent with gel state immiscibility. Both the liquidus and solidus data points deviated from the ideal liquidus and solidus curves respectively for the mixtures of SOPC with DPPC (Figure III-21). Thus suggesting that these lecithins did not exhibit ideal mixing in either the gel or the liquid-crystal. The data in Figure III-21 were obtained using two preparations of SOPC - one containing only 6% of OSPC and

one containing 18% of OSPC. There were no differences in the phase diagrams resulting from the mixtures with the different preparations. This suggested that the presence of small amounts of the wrong positional isomers in mixed-acid lecithin preparations does not substantially affect their mixing behavior.

The phase diagrams obtained with the POPC-DPPC and SOPC-DPPC mixtures could not be fitted to the Lee equations to yield unique values of p_o^{LIQ} and p_o^{SOL}. The derivation of these equations was based on miscibility of the two components in both the gel and the liquid-crystal. The fact that these equations do not fit the data for POPC-DPPC and SOPC-DPPC mixtures was consistent with the presence of substantial immiscibility in the gel state with these mixtures. The phase diagrams were fit to equation II-3 which describes non-ideal mixing in the liquid-crystal assuming that the two components are completely immiscible in the gel. The p_o^{LIQ} value of $0.09 kcal \cdot mol^{-1}$ obtained for mixing of POPC and DPPC indicated that the mixing in the liquid-crystal was nearly ideal as was suggested by the close fit of the data to the ideal liquidus curve in Figure III-22. The curve computed using the value of $0.09 kcal \cdot mol^{-1}$ was shown in Figure III-23. The standard deviation of the residuals of temperature for this fit to the liquidus data was $\pm$ 2.8 degrees (Table III-8). The p_o^{LIQ} value obtained by analysis of the SOPC-DPPC phase diagram was $0.43 kcal \cdot mol^{-1}$. The computed liquidus curve for the SOPC-DPPC mixture using

p_o^{LIQ} = 0.43 kcal·mol^{-1} did not fit the data as well as the POPC-DPPC curves fit (Figure III-25). The standard deviation of the SOPC-DPPC fit was ± 5.8 degrees (Table III-8). The better fit obtained with the POPC-DPPC mixtures was most likely due to the more extensive gel state immiscibility in these mixtures. This made the applications of the analysis used here more appropriate for the POPC-DPPC mixtures than for the SOPC-DPPC mixtures. Total gel state immiscibility was not observed in either of the mixtures, and the analysis used was thus only a good approximation.

The p_o values obtained for these two mixtures supported the idea that small changes in the structure of lecithins affect the way these lecithins mix bilayers in the liquid-crystalline phase. A significant difference in the excess energy of interaction resulted from an increase of two methylene units in the length of the sn-1 chain of the mixed-acid lecithins. In biological systems where the lecithins can have acyl chains that vary in chain length by more than two carbons, the results obtained here would suggest that even greater non-ideality of mixing might occur. The interpretation of the non-ideal behavior observed in the mixtures studied here will be discussed below.

B. Mixtures of DMPC with POPC and PSPC

One of the more interesting occurrences in biological systems is the increase in the unsaturation of the acyl chains in membrane lipids during adaptation to changes in

environmental temperature. To determine some of the effects of insertion of double bonds on the mixing behavior of lecithins in bilayers, the mixing of PSPC and POPC with DMPC was studied. The mixed-acid lecithins contained a palmitate chain at the sn-1 position but the 18-carbon chain at the sn-2 position had a double bond in one lecithin and was saturated in the other. Comparison of the mixing behavior of DMPC with POPC with that of DPPC-POPC mixtures also provided information about the effects of the structure of the single-acid lecithin components on the mixing with a 1-saturated-2-unsaturated lecithin.

The phase diagrams constructed for mixtures of PSPC-DMPC and POPC-DMPC (Figures III-27 and III-28) indicated that these lecithins were miscible in both the gel and the liquid-crystalline states. Attempts to fit the data from these phase diagrams to Equations II-1 and II-2 were made using Method 1 (Section II-7E) but again the ρ_o values from the two equations did not converge on unique solutions. In light of the uncertainties in the ρ_o estimates and the closeness of fit to the data that were found using other procedures for estimating ρ_o values (see below), the lack of unique solutions to the Lee equations was most likely due to the strict tolerance limits placed on the computer fitting routine.

Solution of the Lee equations simultaneously for a series of points on the phase diagrams resulted in ρ_o esti-

mates that varied with the composition of the mixture and the temperature (Figures III-29 and III-30). A large portion of the variability on the ρ_o values estimated by simultaneous solution of the Lee equations was shown to arise from potential measurement errors, especially errors in the temperature measurements due to the broadness of the endotherms (Table III-9).

Liquidus and solidus curves were computed using ρ_o values that were the mean values obtained by simultaneous solution of the Lee equations for $0.2 < X_A < 0.8$. The ρ_o values estimated from the ends of the phase diagrams that had the greatest uncertainty were not included. The computed curves for the two mixtures are shown in Figures III-31 and III-32. For the POPC-DMPC mixtures the ρ_o values were $0.27 kcal \cdot mol^{-1}$ for the liquid-crystal and $0.67 kcal \cdot mol^{-1}$ for the gel (Table III-8). These curves fit the data with standard deviations of residuals of $\pm$ 1.9 degrees and $\pm$ 2.3 degrees for the liquid-crystal and the gel respectively. The ρ_o values for the PSPC-DMPC mixtures were 0.76 and $1.01 kcal \cdot mol^{-1}$ giving standard deviations of the fitted curves of $\pm$ 1.7 degrees and $\pm$ 1.8 degrees for the liquid-crystal and gel respectively (Table III-8).

Estimates of ρ_o values for these mixtures obtained using Method 4 (Section II-7E) are shown in Table III-8. The fits between the data and the computed curves were marginally better using these ρ_o values. The ρ_o values indi-

cated that the mixing was more ideal in the liquid-crystal than in the gel for both pairs of lecithins. In this way, these mixtures were similar to POPC-DPPC and SOPC-DAPC mixtures and to other lipids for which p_o values have been reported (Lee, 1977).

It has been shown that, in binary mixtures of synthetic phospholipids, the miscibility of the lipids in the gel state is related to the differences between their transition temperatures and possibly their structure (Phillips et al., 1970; Chapman et al., 1974; Mabrey and Sturtevant, 1976; van Dijck et al., 1977). The phase diagrams constructed for the mixtures of mixed-acid lecithins with single-acid lecithins studied here are consistent with the previous studies. For pairs of lecithins whic have a large difference in T_c (eg. DPPC with POPC and with SOPC) there was evidence of substantial gel state immiscibility. The phase diagrams for the mixtures of DMPC with POPC and with PSPC suggested limited, if any, gel state immiscibility. The differences in T_c between these lecithins were also less than the differences in T_c between DPPC and POPC or SOPC.

The application of an analysis based on Regular Solution Theory to the phase diagrams for these binary mixtures was used to estimate the excess interaction energies of formation of pairs of unlike molecules. The excess interaction energies for the mixtures in the liquid-crystal were different for each of the lecithin pairs. The data suggest

that the excess interaction energies may be related to differences in the acyl chain length between the lecithins. Only one pair of mixtures allowed a comparison of a change in the unsaturation of the acyl chains (POPC-DMPC and PSPC-DMPC). The ρ_o estimates suggest that the mixing behavior may also be related to the presence or absence of double bonds.

Although there are some uncertainties associated with the application of this type of analysis to the binary mixtures studied here, the ρ_o estimates lead to suggestions about the effects of lipid structure on mixing behavior which can be tested using other lecithin pairs.

Regular Solution Theory attributes the difference in the interaction energies for like molecules and unlike molecules to an enthalpic term. Hence, the excess interaction energies are not directly related to distribution of the molecules in the mixed-lipid bilayers. However, it can be seen intuitively, and it has been noted by Hildebrand and Scott (1964), that a positive excess interaction energy must lead to interactions between like molecules being more energetically favorable than interactions between unlike molecules. Thus mixtures of lipids for which ρ_o is positive would be expected to have transient clusters of like molecules larger than would be predicted for a strictly random distribution (ideal mixing).

Differences in the interaction energy between like molecules and unlike molecules have been related to the size of clusters of like molecules and clusters of unlike molecules by Freire and Snyder (1981) using a statistical approach., These authors have shown computer simulations of "instantaneous" pictures of the distribution of phospholipids in membrane composed of a binary mixture of two lipids. Attempts were made to generate computer simulations based on the excess interaction energies obtained by analysis of the phase diagrams for POPC-SMPC and for PSPC-DMPC. For the mixtures of PSPC with DMPC where ρ_o values were highest, generation of the computer simulation required very long time periods, and during this time, became aware of a potential flow in the computer random number generator involved in the simulation routine. For this reason, no computer simulations are included in this dissertation, since the flow mentioned above might introduce artifacts in any simulations.

D. Biological Implications

Effects of the non-ideal mixing of lipids in membranes, eg. lateral phase separation may be important biologically. It has been known for some time that the activities of some membrane enzymes are affected by the physical state of the phospholipid environment (Mavis and Vagelos, 1972; Esfahani et al., 1972; de Kruyff et al., 1973). The phase separation

of lipids of a particular type in membranes could result in the formation of regions of the membranes which have properties different from the bulk membrane. The physical state of the lipids in these regions might alter the activity of enzymes in these regions, or the formation of such regions might cause changes in the bulk membrane properties that lead to altered enzyme activity.

In addition to the potential effects of lipid clustering on the enzymes in membranes, the permeability of MLVs composed of binary mixtures of saturated single-acid PC to some K^+, ethylene glycerol and water, as well as the fusion of small unilamellar vesicles composed of binary mixtures of saturated SAPC are maximal when the non-ideality of mixing is greatest (Nicolussi et al., 1982). These authors suggest that these findings are related to an increase in the size of fractures on the lipid packing due to the non-ideal mixing of the lipids. Thus non-ideal mixing behavior of lecithins like that observed in this work for mixed-acid PC - single-acid PC systems could have important effects on the biological properties of membranes.

IV-5 Summary

Pairs of fatty acid positional isomers of mixed-acid lecithins containing both saturated and unsaturated acyl chains have been synthesized. Three different synthetic procedures were used, and it was found that the use of the hydrolysis procedure of Keough and Davis (1979) and the

acylation of Gupta *et al.* (1977) resulted in the best yields of mixed-acid lecithins with the least acyl migration.

Differential Scanning calorimetry was used to study the thermotropic behavior of the mixed-acid lecithins having oleate chains and either palmitate, stearate, or arachidate chains and the parent single-acid lecithins. For each pair of positional isomers, the isomers having the 1-long-2-short positional distribution had the lower transition temperature.

Calorimetric studies were also done on mixtures of cholesterol with two pairs of positional isomers. There were quantitative differences in the effect of cholesterol on the thermotropic behavior of these lecithins that suggested a more facile interaction of cholesterol with one isomer than with the other isomer in each isomeric pair. For the SOPC-OSPC pair, more molecules of OSPC were associated with a given amount of cholesterol than was the case for SOPC. For the AOPC-OAPC pair, there were more molecules of AOPC associated with cholesterol than there were OAPC molecules associated with the same amount of cholesterol. The interaction between lecithins and cholesterol would appear to be related to the relative position of the long and short chains in mixed-acid lecithins or to the distribution of the saturated and unsaturated chain or both.

Phase diagrams were constructed for mixtures of DPPC

with POPC and with SOPC and DMPC with POPC and with PSPC. These were analyzed using Regular Solution Theory. The data suggest that differences in the transition temperatures of lecithins affect their miscibility in the gel but not in the liquid-crystal. All mixtures exhibited miscibility in the liquid-crystal while substantial immiscibility was observed in mixtures of DPPC with POPC and with SOPC in the gel phase. Estimates of the excess interaction energies for these mixtures in the liquid-crystal suggested that the structure of the hydrocarbon chains of lecithins can influence the way in which the lecithins are distributed in binary lipid bilayers. The excess interaction energies were used to generate computer simulations and estimates of the number of unlike molecular contacts were obtained.

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APPENDIX A

Computer programs used in this thesis:

Prog. 1	Computation of the liquidus and solidus curves for ideal mixing.
Prog. 2	Simultaneous solution of the equations for non-ideal mixing.
Prog. 3	Computer fit to the equations of Lee (1977, 1978) using method IV (see Materials and Methods).

Prog 1

```
100  PRINT "THIS PROGRAM CALCULAT
     ES THE THEORETICAL CURVES FO
     R IDEAL MIXING OF TWO LIPIDS
     "
200  PRINT "YOU MUST ENTER THE TR
     ANSITION TEMPERATURES & ENTH
     ALPIES OF THE PURE COMPONENT
     S"
300  PRINT "THE COMPUTER WILL OUT
     PUT VALUES FOR X-LIQ & X-SOL
      FOR VARIOUS T'S"
400  PRINT "PROGRAM WRITTEN BY P.
      J. DAVIS"
500  PRINT " ": PRINT "
     **********": PRINT
     " ": PRINT " "
600  PRINT "WHAT IS TRANSITION TE
     MPERATURE FOR HIGHER MELTING
      COMPONENT??? "
700  INPUT TA
800  PRINT "WHAT IS THE TRANSITIO
     N TEMPERATURE OF LOWER MELTI
     NG COMPONENT??? "
900  INPUT TB
1000  PRINT "WHAT IS ENTHALPY OF
     HIGHER MELTING COMPONENT???
     ": INPUT HA
1100  PRINT "WHAT IS ENTHALPY OF
     LOWER MELTING COMPONENT??? "
     ;: INPUT HB
1150  PRINT "TEMP                X-LIQ
                X-SOL"
1160  PRINT "-------------------
     ------------------"
1200 I = 1
1300  FOR T = TB TO TA STEP 0.5
1400 R = (HB / 1.987) * ((1 / T) -
     (1 / TB))
1500 S = (HA / 1.987) * ((1 / T) -
     (1 / TA))
1600 XL = (1 -  EXP (R)) / ( EXP
     (S) -  EXP (R))
1700 XS = XL *  EXP (S)
1800  PRINT T;"    ";XL;"    ";XS
1900  NEXT T
1950  PRINT "IF YOU WISH TO CONTI
     NUE TYPE 'YES' ";: INPUT Z$
1960  IF (Z$ = "YES") GOTO 500
1987  PRINT "          **********
     **********"
2000  END
```

Prog 2

```
10  PRINT "PROGRAM TO COMPUTE RO'
    S BY SIMULTANEOUS SOLUTION O
    F LEE EQUATIONS"
20  PRINT " OPERATOR MUST INPUT I
    N THIS ORDER : T-A, T-B, H-A
    , H-B, T, X-LIQ, X-SOL"
30  PRINT "OUTPUT IS THE RO-LIQ A
    ND RO-SOL"
40  PRINT "----------------------
    ------------------"
41  PRINT "**********************
    ******************": PRINT "
    ": PRINT " "
42  PRINT "PROGRAM WRITTEN BY P.
    J. DAVIS"
43  PRINT " ": PRINT "**********
    ****************************
    * ": PRINT "-----------------
    --------------------------"
100   INPUT TA,TB,HA,HB
200   INPUT T,XL,XS
300 C1 = (HB * T) / TB - HB - (2 *
    T * LOG ((1 - XL) / (1 - XS
    )))
400 C2 = (HA * T) / TA - HA - (2 *
    T * LOG (XL / XS))
500 A2 = (1 - XL) ^ 2
600 A1 = XL ^ 2
700 B2 = (1 - XS) ^ 2:B1 = XS ^ 2

800 F = B1 / B2
900 A3 = A2 * F
1000 C3 = C2 * F
1100 AD = A1 - A3
1200 CD = C1 - C3
1300 LRO = CD / AD
1400 Y = A1 * LRO
1500 Z = C1 - Y
1600 SRO =  - (Z / B1)
1700  PRINT "RO-LIQ=> ";LRO,"RO-S
    OL=> ";SRO
1750  PRINT "EVALUATE AT MORE POI
    NTS?? TYPE YES ": INPUT G$
1760  IF (G$ = "YES") GOTO 200
1800  PRINT "IF THERE ARE NEW PAR
    AMETERS FOR PURE CPDS....TYP
    E N>": INPUT R$
1900  IF (R$ = "N") GOTO 100
2000  END
```

Prog 3

```
1  PRINT "THIS PROGRAM CALCULATES
     THE VALUES OF X-LIQ & X-SOL
     FOR A GIVEN T"
2  PRINT "PROGRAM INITIALLY ASKS
     FOR T'S AND H'S FOR THE PURE
     LIPIDS AND THE NONIDEALITY
     FACTORS (CAL.)"
3  PRINT "IT THEN ASKS FOR THE T
     FOR WHICH YOU WISH THE X-LIQ
     AND X-SOL"
4  PRINT "YOU HAVE THIS PROGRAM C
   OURTESY OF P. J. DAVIS"
6  PRINT " "
7  PRINT "**********************
   ******************"
8  PRINT "          ************
   *******"
9  PRINT "   "," "
10 PRINT "WHAT IS TEMPERATURE OF
     TRANSITION OF HIGHER MELTIN
     G COMPONENT?";
20  INPUT TA
30  PRINT "WHAT IS ENTHALPY OF TR
     ANSITION OF HIGHER MELTING C
   OMPONENT? ";
40  INPUT DHA
50  PRINT "WHAT IS THE TEMPERATUR
     E OF TRANSITION OF LOWER MEL
     TING COMPONENT? ";
70  INPUT TB
80  PRINT "WHAT IS THE ENTHALPY O
     F TRANSITION OF LOWER MELTIN
     G COMPONENT? ";
90  INPUT DHB
100  PRINT "ENTER LIQUID RO ! ";
101  INPUT LRO
102  PRINT "ENTER SOLIDUS RO ! ";

103  INPUT SRO
108  PRINT "FOR WHAT TEMPERATURE
     DO YOU WISH TO EVALUATE THE
     COMPOSITION ? ";
109  INPUT TC
110 XL = 0.5
112 XS = XL + (0.1)
2000  GOSUB 7000
2050 T1 = T
2100  GOSUB 8000
2150 T2 = T
2200  PRINT TC;"   ";T1;"  ";T2
2210  PRINT "IF SATISFACTORY, TYP
     E OK"
2220  INPUT V$
2230  IF (V$ = "OK") GOTO 5000
```

```
2250  PRINT "NEW  XL/XS"
2300  INPUT XL,XS
2350  GOTO 2000
5000  PRINT "T= ";TC,"X-LIQ= ";XL
     ,"X-SOL= ";XS
5010  PRINT "RO-LIQ = ";LRC,"RO-S
     OL =.";SRO
5020  PRINT "NEW RO'S ??? TYPE YE
     S";
5025  INPUT W$
5030  IF (W$ = "YES") GOTO 100
5050  PRINT "IF STOP. TYPE END"
5100  INPUT Z$
5150  IF (Z$ = "END") GOTO 9999
5200  GOTO 108
7000 A = ((((1 - XL) ^ 2) * LRO) -
     (((1 - XS) ^ 2) * SRO)) / 1.
     987
7010 B = DHA / 1.987
7020 C = (DHA / (1.987 * TA)) -  LOG
     (XL / XS)
7030 T = (A + B) / C
7040 F =  ABS (T - TC)
7050  RETURN
8000 A = (((XL ^ 2) * LRO) - ((XS
     ^ 2) * SRO)) / 1.987
8010 B = DHB / 1.987
8020 C = (DHB / (1.987 * TB)) -  LOG
     ((1 - XL) / (1 - XS))
8030 T = (A + B) / C
8040 F =  ABS (T - TC)
8050  RETURN
9999  PRINT " ": PRINT "I HOPE TH
     IS PROGRAM WAS HELPFUL": PRINT.
     "**********************
     *****"
10030  END
```